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## Maternal Ethanol Consumption: Glycoproteins and Gangliosides of the Central Nervous System in the Rat Offspring

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MATERNAL ETHANOL CONSUMPTION:  
GLYCOPROTEINS AND GANGLIOSIDES  
OF THE CENTRAL NERVOUS SYSTEM  
IN THE RAT OFFSPRING

by

Antonio Blavatsky Caesar Noronha

A Dissertation Submitted to the Faculty of the Graduate  
School of Loyola University of Chicago in Partial  
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of

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## VITA

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## LIST OF ABBREVIATIONS

Abbreviation	Identity
ADH	Alcohol dehydrogenase
BS	Brain stem
CB	Cerebellum
CNS	Central Nervous System
CPM	Counts per minute
CS	Corpus striatum
CX	Cerebral cortex
DPM	Disintegrations per minute
EEG	Electroencephalograph
FAS	Fetal Alcohol Syndrome
FUC	Fucose
GABA	$\gamma$ -aminobutyric acid
GAL	Galactose
Gal NAc	N-acetyl-galactosamine
Glc NAc	N-acetyl-glucosamine
HC	Hippocampus
HPTLC	High performance thin-layer chromatography
HSA	Human Serum Albumin
HT	Hypothalamus
m.a.	milliamperes
MAN	Mannose
NAD	Nicotinamide adenine dinucleotide

## LIST OF ABBREVIATIONS

Abbreviation	Identity
NANA	N-acetyl-neuraminic acid
nm	Nanometers
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
rpm	Revolutions per minute
SA	Sialic acid
SDS	Sodium dodecyl sulphate
SM	Sphingomyelin
SPM	Synaptic plasma membrane
TEMED	Tetramethylethylenediamine
TLC	Thin-layer chromatography

CHAPTER I  
BACKGROUND AND  
LITERATURE REVIEW

Introduction

Fetal Alcohol Syndrome (FAS) is a disorder which is frequently seen in the offspring of mothers who are chronically alcoholic. This disorder is characterized by prenatal and postnatal growth deficiencies, microcephaly and numerous craniofacial, skeletal, cardiac and genital abnormalities. Central nervous system dysfunction and irreversible mental retardation are however the most serious defects of this syndrome.

The purpose of the research reported in this dissertation is to gain an insight into some of the potential neurochemical causes of mental retardation associated with the fetal alcohol syndrome. As previous work strongly suggests that CNS abnormalities frequently associated with FAS children may be related to alterations in central nervous system (CNS) membranes, the research reported in this dissertation will examine the effects of alcohol on the synthesis of the functional components of the synaptic plasma membranes, namely the glycoproteins and gangliosides. These studies will be performed during

central nervous system development and during the period of active synaptogenesis in particular.

### Development of the Central Nervous System

During development, the CNS presents a constantly changing histological picture. However, the process of CNS development from the earliest embryological period through fetal, neonatal and young adult life shows a remarkable similarity from one species to another. Hence, for the ease of discussion and organization, events of the nervous system development have often been arbitrarily divided into different stages.

It should be noted, however, that the nervous system is heterogeneous from region to region in the timing of development of cell types and also the complexity of interaction among those types.

The four main stages of development have been defined as:

- I. Organogenesis and neuronal multiplication.
- II. The brain 'growth spurt' sometimes termed the 'critical period' which includes:
  - A. A maturation period of axonal and dendritic growth.
  - B. Synaptogenesis which is followed by glial multiplication and myelination.
- III. Mature, adult size.



#### IV. Aging or the period of senile regression.

Research included in this dissertation mainly focuses on the period of synaptogenesis or Stage IIb of development. The time of stress (exposure to alcohol) is during the period of organogenesis and neuronal multiplication. Hence, the morphological and biochemical events associated with these two stages of development shall be elaborated upon in more detail than the other developmental stages.

#### Stage I: Organogenesis and Neuronal Multiplication

The initial events leading to the formation of the nervous system have been described in detail (Crelin, 1974; Balinsky, 1975 and Jacobson, 1978). In rat, this stage encompasses the time until birth. This is the period of the embryonic and fetal development of the brain from an axial neural or medullary plate which subsequently folds and forms an elongated hollow structure called the neural tube. From the head end of this tube three prominent swellings emerge, which form the three main parts of the brain namely the forebrain, the midbrain and the hindbrain -- in other words neural induction takes place. The central lumen enclosed by the walls of the neural tube forms the small, apparently functionless central canal of the adult spinal cord and the much more important ventricular system of the brain.

Lining the lumen is a collection of primitive,

embryonic dividing cells from which are formed the spongioblasts which are precursors of glia, and the neuroblasts, which are precursors of neurons. At this early period of organogenesis, the process is one of differential growth, accompanied by cell division and migration within the tissue in an orderly sequence.

During the migration of the neuroblasts, four histologically different layers develop in the wall of the neural tube as here described for the telencephalon.

- a. The marginal zone initially contains only cytoplasmic processes of cells whose nuclei are at deeper levels, but later on becomes sparsely populated by neurons that form layer I of the six layered (laminae I-VI) cerebral neocortex which is the first cortical layer to develop.
- b. The intermediate zone develops as a result of an ingrowth of afferent axons and migration of cells and ultimately forms the cortical plate. Cells and fibers continue to invade this zone resulting in the cortical plate undergoing progressive differentiation to form the definite layers of the cerebral cortex.
- c. The subventricular zone contains proliferating cells that mainly give rise to macroglia (oligodendrocytes and astrocytes) and to the smaller Golgi type II neurons.
- d. The ventricular zone contains the ventricular germinal cells and is the first zone to develop. From it originates the other zones and then it disappears at an early stage

of development. The germinal cells in the ventricular zone give rise to the large Golgi type I neurons.

Young neurons are generated continuously by mitosis of germinal cells close to the ventricle and begin to move outwards as a continuous stream. Angevine and Sidman (1961) found that young neurons labelled with tritiated thymidine on day 11 in utero migrate from the ventricular germinal zone to the deep layer of the occipital cortex, while cells labelled on subsequent days migrate to more superficial layers of the cortex.

This "inside-out" sequence of arrival of neurons in the cerebral isocortex has also been observed in the rat fetus by Berry and Rogers (1965) and Berry et al. (1964a,b). They demonstrated that young neurons that are generated on intrauterine day 16 in the cerebral cortex of the rat fetus migrate out to the subpial level and differentiate there. Neurons formed on days 17-22 of gestation migrate between the cells of the previously formed layers to form new layers superficial to them. Thus, layer IV has been shown to originate on day 16 and cells that form layer II originate last on day 21 of gestation. The neurons that arrive in the cortex first are displaced to deeper levels by those that arrive later. Thus in the cerebral isocortex, the neurons of the outer layers (except I) originate last and are the most recent to have appeared in evolution. Layers I and VI appear to have evolved first and are the first

cortical layers to develop.

In any part of the nervous system, large neurons are produced before smaller neurons. The smaller neurons are invariably local circuit neurons such as granule cells of the cerebellum or Golgi type II neurons. The pioneering work of Ramon y Cajal in the nineteenth century has demonstrated that in the cerebellar cortex the Purkinje cells are formed first and then the Golgi type II cells followed by basket and stellate cells, and finally the granule cells.

Thus, we see that stage I of central nervous system development is characterized by a differentiation and proliferation of cells. During this period, the number of cells in the rat brain reaches 94-97% of the adult number.

Biochemically, this early proliferation of cells is indicated by synthesis of DNA. Altman (1969) and Brasel et al. (1970) have demonstrated that the rate of incorporation of radioactive thymidine into DNA and the activity of DNA polymerase increase during periods of cell proliferation in a given region and then decrease as cell division ends.

Tettamanti (1971) has observed a three-fold increase in the content of ganglioside, namely lipid bound sialic acid, during morphogenesis in rat, i.e. from the 8th day prenatally to birth. They have attributed this increase of gangliosides in rat brain during the embryological and fetal stages of development to the occurrence of two main events:

1. The increase in the number of neurons, occurring during the same period of life.
2. The accumulation of gangliosides inside the individual neurons as has been demonstrated by Garrigan and Chargaff (1963).

Stage IIa: Maturation Period of Axonal and Dendritic Growth

This is a postnatal stage in rat and occurs from birth until approximately ten days postnatally. At birth, in rat and mouse, the CNS contains mainly neurons in the early stages of growth, some neuroglia, but no myelin. During Stage IIa the brain increases in weight, neurons increase in size and especially in the length of their axons. There is also an increase in the number, size and arborization of their dendritic processes. This neuronal growth increases the volume of each neuron several-fold. In early development neurons are mainly somas showing little or no process formation but as development proceeds, the somas become quantitatively less important and the outgrowth or processes represent the major part of the neuronal material. However, at the same time, the number of neurons remains constant while the weight of the brain increases five- or six-fold. Thus, the number of neurons per unit volume actually decreases in an inverse ratio to the increase in weight of the CNS.

Eayrs and Goodhead (1959) have studied the postnatal development in the cerebral cortex of rat. At birth, local cortical differentiation has barely commenced and the cells are small and closely packed. Of the several laminae, only layer I

can be distinguished easily, while layers II and IV cannot be separated and consist of cells which are strongly chromophilic and which are arranged in orderly columns. The position of a developing layer V can however be identified as a band of somewhat larger neurons; while layer VI is represented by a zone of cells in which the Nissl substance is scanty and whose arrangement lacks any of the orderly pattern of those in layers III and IV. Very few axons can be seen in the outermost layers of the cortex; and although considerably more are present in the deeper layers of the cortex, most of these fibers run a tangential course and no radially orientated bundles of axons are visible. However, during Stage IIa of development increased cortical differentiation occurs and the six layers of the cortex begin to be recognized without difficulty. The appearance of the cortex is generally characterized by an increase in the size of the perikarya, a reduction in the packing density and a more precise demarcation between the several laminae. The boundary between layers III and IV shows the presence of larger cells which are apparently destined to develop into the 'border pyramids' as seen in the adult. Layer IV displays its characteristic granular appearance and there is a marked pyramidization of cells in layer V and a few cells also possess the argyrophilic properties of the adult. The cells of the infragranular layers have also developed conspicuous amounts of Nissl substance. A marked increase

in axon density has also occurred particularly in the granular and infragranular layers where many radially orientated fibers are now present, though not conspicuously assembled into fasciculi. Also, considerable numbers of the apical dendrites now extend into layer I and numerous basal dendrites are present.

Biochemically, during this stage of development the ratio of protein to DNA which is a good indicator of cell size begins to increase which reflects in part, the arbo- rization of neuronal processes (Benjamins and McKhann, 1981). As DNA content peaks, the ratio of RNA to DNA begins to increase. Most of the newly synthesized RNA remains in the nucleus until cell division has ended. Upon differen- tiation, the nucleolus matures, with increased RNA synthesis, especially of ribosomal RNA (rRNA) followed by a transfer of RNA into the cytoplasm. In neurons, Nissl substance (rRNA) first becomes prominent at this stage. Hyden (1943) and Guiffrida et al. (1975) have shown that the rate of RNA synthesis decreases after maturation together with the activity of RNA polymerase in nuclei of both neurons and glia.

During maturation of rat brain, Margolis and Margolis (1977) have shown that the levels of the glycosaminoglycans hyaluronic acid, chondroitin sulfate, and heparin sulfate increase after birth, reach a peak during Stage IIa of development and then decline to adult levels by about 30

days. Hyaluronic acid shows the most dramatic change, decreasing from 3.6  $\mu\text{mol}$  hexosamine per gram of lipid-free dry weight at 8 days to 1.4  $\mu\text{mol}$  at 12 days. In addition, they found that almost 90 percent of the hyaluronic acid is soluble at 7 days. They speculate that the high levels of soluble hyaluronic acid in the immature brain may function in the extracellular space to retain water and form a penetrable matrix for cell migration. It should be noted also that neuronal cell bodies generally have higher levels of glycosaminoglycans than do glial cells.

#### Stage IIb: Synaptogenesis Which is Followed by Glial Multiplication and Myelination

This stage ranges approximately from the 10th until the 20th day postnatally. During this stage two very important events in the maturation of the brain -- synaptogenesis and myelination -- begin to occur. Synaptogenesis slightly precedes myelination though both processes show a great degree of overlap with each other.

Synaptogenesis is one of the most critical and important events that takes place in the development of the brain requiring an interaction between the pre- and post-synaptic elements. One striking aspect of this period is the consistent timing in the appearance of synapses -- less than a day in time between individuals.



Synapses appear very suddenly and then show a rapid increase in numbers. This dramatic increase in neuronal connections is not a random event but rather a very selective one (Jacobson, 1978). Studies of Voeller et al. (1963) and Meller et al. (1968) have shown that axodendritic synapses precede the formation of axosomatic synapses. In addition, there is strong evidence which indicates that the formation of these connections is governed by genetic and developmental processes that function without any influences from the external environment.

Many investigators (Von Economo, 1926; Peters and Flexner, 1950; Shariff, 1953; Sholl, 1953) have used the cell-to-gray coefficient as an index of connectivity or functional capacity in the developing cerebral cortex. Another estimate of neuronal connectivity has also been obtained by accurate counts of the frequency of synaptic profiles obtained in electron microscopic sections stained with ethanolic phosphotungstic acid which selectively stains synaptic junctions (Bloom, 1972; Jones, 1973; Jones et al., 1974). All these studies have revealed a great increase in the neuronal connectivity of the mammalian cerebral cortex in the early postnatal period of development. Eayrs and Goodhead (1959) have estimated that a ten-fold increase in connectivity occurs between days 12 and 30 after birth in the cerebral cortex of the rat. Aghajanian and Bloom (1967) have calculated a seven-fold increase, amounting to

$12 \times 10^8$  synapses per cubic millimeter, in the molecular layer of the rat parietal cortex from the 12th to the 26th day after birth.

Anatomically, the cerebral cortex of the rat has assumed the features characteristic of the adult during this phase. All six layers are well marked and the maximal increases in the densities of the axons and the dendrites are seen during this phase, resulting in the establishment of the neuronal circuitry (Jacobson, 1978). The Purkinje cell, which is the principal neuron and the sole neuronal pathway out of the cerebellar cortex, receives impulses directly from the climbing fibers which originate in the inferior olive and indirectly by the mossy fibers which are mostly the terminals of spinocerebellar and pontocerebellar fibers. The afferent climbing fibers synapse on the large dendritic branches of the Purkinje cells (Larramendi and Victor, 1967) while the mossy fibers terminate in structures called glomeruli in which they form synapses with dendrites of granule cells and the axons of the Golgi type II cells. The granule cell thus receives an input from the mossy fiber. The granule cell axon (also known as the parallel fiber) forms synaptic connections on dendritic spines of a row of Purkinje cells, and as well synapse on the dendrites of the stellate, basket and Golgi cells. The stellate cells in turn form

synapses on Purkinje cell dendritic shafts, while the basket cell makes synapses with the soma of the Purkinje cell. The Golgi cell dendrites on the other hand receive inputs from parallel fibers, while the Golgi cell axon synapses with the granule cell dendrites within the glomerulus.

Biochemically, maturation of such elements as neuronal processes, synaptic endings and myelin sheaths which occurs in this stage involves the deposition of lipids and proteins into those membraneous structures. As with most other constituents, proteins increase most rapidly in rat cortex during the first two to three weeks of postnatal life. In this same period, there is a shift from water-soluble to membrane-bound proteins. Compositions of the protein populations also change during development.

Bignami and Dahl (1974) have shown that glial fibrillary acidic protein which is localized in astroglial processes shows a peak concentration at 10 to 14 days in mouse brain, thus reflecting astroglial differentiation at the time of myelination (Jaque et al., 1976). Other brain-specific proteins also appear about the time myelination begins -- the acidic S-100 protein which is primarily glial and the basic and proteolipid proteins of myelin.

Activities of a number of enzymes involved in metabolism of myelin lipids increase during this period of most rapid myelination and then decrease as the rate of myelin accumulation slows. These include 2',3'-cyclic nucleotidase (Kurihara and Tsukada, 1968), a cholesterol ester hydrolase active at pH 7.2 (Eto and Suzuki, 1973), a protein kinase (Miyamoto and Kakiuchi, 1974), a phosphatase (Miyamoto and Kakiuchi, 1975) and the enzyme UDP-galactose-ceramide galactosyl transferase which synthesizes galactocerebroside (Neskovic et al., 1973).

Lipid content of the brain increases rapidly during this period and is closely related to the onset of myelination. Galactocerebroside, cerebroside sulfate, sphingomyelin and triphosphoinositide which are characterized by low concentrations at three days postnatally show a dramatic increase between 12 and 18 days in keeping with their high degree of localization in the myelin membrane. The activity of the enzymes that synthesize galactocerebroside and cerebroside sulfate parallels myelination, showing peaks of activity at about 17 to 20 days (McKhann and Ho, 1967; Costantino-Ceccarini and Morell, 1971).

Gangliosides increase very rapidly during this period and reach about 90 percent of the adult value by day 24. The pattern of gangliosides changes with GD<sub>1a</sub> as the predominant ganglioside as the mature brain (Vanier et al.,

1971). Increases in gangliosides and in the activity of the enzyme that synthesizes their precursor, glucocerebroside coincide with the outgrowth of axons and dendrites which is in agreement with the possible localization of much of the ganglioside in nerve processes.

Thus, elevation of the galactolipid and plasmalogen content of the brain correlates well with the morphological appearance of myelin, and increases in gangliosides appear to be related to the increasing arborization of neurons.

### Stage III: Mature, Adult Size

This period begins about the 20th day postnatally in rat and lasts until full maturation of the brain is reached.

### Stage IV: Aging or the Period of Senile Regression

This period is associated with the general aging and senile regression of the brain.

### Synapses

Synapses are highly specialized junctions between nerve cells and are present in all animals possessing a nervous system. The first descriptions of synaptic ultrastructure were made by Sjostrand (1953), Palade (1954), Palay (1954, 1956) and De Robertis and Bennett (1954, 1955). From these studies it emerged that synapses

consisted of pre- and post-synaptic neuronal elements, each possessing a 7-10 nm thick membrane which were separated from each other at the synaptic junction by a 10-20 nm cleft.

Synapses fulfill the role of establishment and construction and reinforcement of neuronal pathways of communication and transmission.

Synapses are generally either electrical or chemical in nature. Electrical synapses are predominant in invertebrates and are formed by actual physical contact of the plasma membranes of the two adjacent neurons. On the other hand, chemical synapses form the basis of nervous control in vertebrates. They contain a 100-300 Å gap and impulses are transmitted across adjacent neurons through this gap only by releasing a neurotransmitter stored in the pre-synaptic nerve ending. This chemical substance or transmitter diffuses across the cleft and reacts with the post-synaptic receptors thereby triggering a new impulse. Chemical synapses possess several key properties, such as delay, rectification, summation and inhibition, which the nervous system uses in an impressively large number of ways in its control and processing of impulses (Eccles, 1963).

The structure of the pre- and post-synaptic membranes has attracted much attention among neurochemists in recent years for it must account for the mechanism of

neurotransmitter release, the binding to receptors on the post-synaptic membranes and the subsequent generation of action potentials.

Many years ago, it was demonstrated that homogenization of brain tissue in an isotonic medium resulted in the nerve terminals being sheared off from their axonal connections and from surrounding glial elements and that these terminals were resealed to form synaptosomes. The synaptosomes contained the pre-synaptic membrane, the intersynaptic cleft and part of the post-synaptic membrane (De Robertis, 1969; Whittaker, 1969). More recently, synaptic plasma membranes (SPMs) or membranes freed from the internal contents of nerve endings have been isolated with a high degree of purity from several sources (Morgan et al., 1971; Cotman and Matthews, 1971; Gurd et al., 1974; Babitch et al., 1976 and Smith and Loh, 1977). The isolation of synaptic plasma membranes has presented scientists with a unique opportunity of studying the biochemical characteristics of neuronal membranes with minimum glial contamination and the purification of SPM has permitted an extensive analysis of its structural and chemical properties.

#### Isolation of Synaptosomes and Synaptic Plasma Membranes

A major advance in biochemical studies of synapses was made when Gray and Whittaker (1962) and De Robertis et al. (1962) independently demonstrated that particles which looked like intact nerve endings were abundant in a

crude mitochondrial fraction obtained from the brain. Fractionation of this crude mitochondrial fraction on a discontinuous sucrose gradient (Whittaker et al., 1964) resulted in myelin being separated above the 0.8M sucrose layer, synaptosomes being concentrated at the 0.8-1.2M interface and mitochondria being concentrated as a pellet. Following this successful preparation of a highly enriched synaptosomal fraction, interest was then directed towards other components of the synaptosome namely the synaptic plasma membrane (Rodriguez de Lores Arniaz et al., 1967; De Robertis et al., 1967a; Cotman et al., 1968a, 1968b; Koch, 1969; Cotman and Matthews, 1971; Morgan et al., 1972; Gurd et al., 1974), the synaptic junctional complex (De Robertis et al., 1967a; Kornguth et al., 1969, 1971; Sellinger et al., 1972) and the post-synaptic membrane (Garey et al., 1972).

There has also been a proliferation in the number of techniques at the disposal of investigators. Thus, while initial and many later studies employed only discontinuous sucrose gradients (Gray and Whittaker, 1962; De Robertis et al., 1962a, 1963), a wide range of techniques have been adapted for use with brain tissue. Among these are continuous sucrose gradients (Whittaker, 1968a), continuous and discontinuous Ficoll gradients (Kurokawa et al., 1965a; Adbel-Latif, 1966; De Robertis et al., 1967a; Autilio et al., 1968; Flexner et al., 1971; Cotman and Matthews, 1971; Haga, 1971



and Garey et al., 1972), caesium chloride gradients (Kornguth et al., 1967, 1969, 1971, 1972) and colloidal silica gradients (Largercrantz and Pertoft, 1972). Other techniques include zonal centrifugation (Mahaley et al., 1968; Cotman et al., 1968a,b; Mahler and Cotman, 1970 and Festoff et al., 1971) and Millipore filters (Baldessarini and Vogt, 1971) for the preparation of subcellular fractions. It should be noted, however, that synaptic plasma membranes obtained directly from an osmotically shocked crude mitochondrial fraction seems to be contaminated by intracellular membranes (Cotman et al., 1968). Thus, there is no certainty as to the identity and homogeneity of the plasma membranes, as the crude mitochondrial fraction contains a variety of vesicular elements bound by a plasma membrane and a number of free plasma membranes which include axonal, dendritic and glial fragments. In addition, Cotman et al. (1971b) have found that a portion of the plasma membrane from glial cells sediments in the crude mitochondrial fraction and has an isopycnic banding density very similar to that of synaptic plasma membranes and intact synaptosomes on sucrose gradients. Lemkey-Johnston and Dekirmenjaih (1970) have also reported that axonal membranes constitute a major source of membranes in crude mitochondrial fractions and, like glial membranes have an isopycnic density very similar to synaptic plasma membranes and synaptosomes.

In order to circumvent these difficulties and to

further reduce intracellular membrane fragments, it seems almost essential to prepare synaptic plasma membranes directly from synaptosomes. The major improvement in preparation of synaptosomes in recent years has been the use of Ficoll rather than sucrose density gradients (Morgan et al., 1971; Cotman and Matthews, 1971). However, one of the contaminants still likely to be present in synaptosomes prepared by Ficoll gradient centrifugation are membranes derived from the rough endoplasmic reticulum. Recently, several improvements have been made in order to get rid of this source of contamination. The method of Gurd et al. (1974), which is similar to the method of Cotman and Matthews (1971), suggests that three additional washes of the crude mitochondrial pellet with 0.32M sucrose significantly reduces the contamination with membranes of the endoplasmic reticulum. In addition, they suspend the washed crude mitochondrial pellet in 14% Ficoll and overlay it with 7.5% Ficoll and centrifuge the gradient. The synaptosomes float up to the interface between the two Ficoll layers and the mitochondria collect as a pellet at the bottom. This modification reportedly reduces mitochondrial contamination.

The preparation of synaptic plasma membranes from synaptosomes has been improved by including a step where synaptosomes are lysed in an alkaline hypotonic media. According to Cotman and Matthews (1971), this modification gives a more efficient release of synaptic "ghosts" and thus

results in a better separation of synaptic plasma membranes from mitochondria in the sucrose density gradient.

### Chemical Composition of SPM

Synaptic plasma membranes have been isolated from several species, but those isolated from rats have been extensively characterized (Morgan et al., 1971; Cotman and Matthews, 1971; Gurd et al., 1974). Like all other biological membranes, SPM are composed primarily of proteins and lipid with carbohydrates bound to components of each. Relative to the concentration of protein, the amounts of both lipid and carbohydrate are significantly higher than the corresponding ratios of those in plasma membranes from most non-neuronal cells. Since the insulatory properties of membranes are conferred by lipids, the high lipid to protein ratio may reflect the importance of maintaining a high electrical resistance in the nerve membrane during its resting state. On the other hand, the high percentage of carbohydrate almost certainly signifies an abundance of receptors, cell-recognition sites and other highly specific moieties (Brunngraber, 1969; Irwin, 1974).

### Proteins and Glycoproteins

SPM contain a wide variety of polypeptide species. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate

(SDS) of SPM has revealed as many as twenty Coomassie Blue sensitive bands (Banker et al., 1972; Morgan et al., 1973; and Gurd et al., 1974). However, many more species probably exist but are not observed because they are present in amounts too small to be detected by the available staining methods or because they co-migrate with visualized species. Three major species or bands in SPM have consistently been observed having molecular weights of 95,000, 50,000 and 40,000, respectively. The ~95,000 molecular weight protein is believed to be the subunit of  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$  (Morgan et al., 1973) while the 50,000 and 40,000 molecular weight species are almost certainly the structural proteins tublin and actin, respectively (Blitz and Fine, 1974; Wang and Mahler, 1976).

Synaptic plasma membranes contain a number of glycoproteins. This has been demonstrated by labelling the membrane in vivo with [ $^3\text{H}$ ]-fucose (Gurd and Mahler, 1974; Smith and Loh, 1977b) or in vitro with galactose oxidase (Wang and Mahler, 1976; Smith and Loh, 1977b) and by direct staining of gels with Schiff reagent (Gurd and Mahler, 1974; Zanetta et al., 1975 and Smith and Loh, 1977b) and also by means of affinity chromatography on Con A-sepharose (Gombos et al., 1974; Zanetta et al., 1975, 1976). All these techniques have revealed a large number of species and have demonstrated the presence of carbohydrate in most of the Coomassie Blue-sensitive polypeptides. However,

extraction of the membrane with sodium hydroxide has demonstrated that much of the Coomassie Blue-sensitive polypeptides can be removed from the membrane without any loss of the Schiff-staining material, thus providing direct proof that many of the bands are in fact heterogenous in nature.

Mena and Cotman (1982) have reported that some of the synaptic membrane glycoproteins are similar in their amino acid composition but differ only in their carbohydrate sequence, composition or points of attachment of the carbohydrate to the protein. This suggests that the specificity of the glycoprotein may be due to the carbohydrate structure, as the amino acid sequences of these glycoproteins have a high degree of homology.

Fucosyl glycoproteins are the major glycoprotein class in synaptic plasma membranes and have been shown to contain more than 26% of protein and about 85% of the protein-bound sugar associated with these membranes. Zanetta et al. (1977) separated synaptosomal plasma membrane glycoproteins by sequential affinity chromatography on immobilized lectins and reported the presence of more than twenty bands in fucosyl-glycoprotein fractions. Gurd (1979) studied the incorporation of labeled fucose into synaptic membranes, synaptic junctions and post-synaptic densities and found that the relative specific activity of the labelled fucose associated with the synaptic membranes was higher than that of the synaptic junctions and the post-synaptic densities. Recently,

Fu et al. (1981) studied the development of fucosylated synaptic membrane glycoproteins in cortices of developing rats. They reported that labelled fucose was incorporated into most of the synaptic membrane glycoproteins and that a major portion of the radioactivity was associated with the higher molecular weight glycoproteins. In addition, they noted that the incorporation of [ $^3\text{H}$ ]-fucose into synaptic fractions decreased two- to three-fold between 10 and 28 days of age and that the relative synthesis of the lower molecular weight glycoproteins showed a marked increase with age.

In addition to fucose, glycoproteins of SPM also contain several other hexoses which include glycosamine, mannose as well as sialic acid (Gombos et al., 1972). This has been substantiated by studies in which detergent-solubilized SPM glycoproteins were fractionated on lectin-affinity columns and which bind specific carbohydrates (Gurd and Mahler, 1974; Zanetta et al., 1975). Finally, many of the glycoprotein species in SPM have been shown to be sulfated (Simpson et al., 1976).

### Lipids

The lipid component of SPM consists primarily of phospholipids, cholesterol and gangliosides. Lesser amounts of several other lipid species are also present. Galactocerebro-sides which are characteristic of and believed to be exclusive

of brain tissue are virtually absent from SPM (Breckenridge et al., 1972). The primary phospholipids in SPM are phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC) and sphingomyelin (SM). The molar sum of (PE + PS) is approximately equal to that of (PC + SM) and thus SPM adheres to the rule first formulated by Bretscher (1973) that postulates an equal number of amino-containing and choline-containing phospholipid molecules in biological membranes. The other major lipids in SPM are cholesterol and the gangliosides. The cholesterol/phospholipid ratio for SPM is lower than that of most other plasma membranes (Breckenridge et al., 1972), while its ganglioside content is considerably higher.

### Enzymes

SPMS are rich in a great many enzyme activities, including both those characteristic of all or most other plasma membranes, such as  $(\text{Na}^+, \text{K}^+)$ -ATPase, 5'-nucleotidase and adenylate cyclase, as well as those largely restricted to nervous tissue such as acetylcholinesterase and neuraminidase (Brunngraber, 1979). SPMs also contain receptors for neurotransmitters or putative neurotransmitters such as acetylcholine, glycine and  $\gamma$ -aminobutyric acid (GABA), as well as for opiate drugs (Smith and Loh, 1979). In addition the membrane possesses receptors for several different types of lectins (Zanetta et al., 1978; De Silva,

1979).

### Orientation of SPM Components

According to our current understanding, biological membranes are composed of a fluid lipid bilayer into which protein molecules are embedded (Singer and Nicolson, 1972). Singer (1974) has proposed that membrane proteins are generally of two types -- peripheral, which are superficially attached to the lipid bilayer by electrostatic bonds and integral proteins which are deeply embedded in the lipid bilayer and held primarily by hydrophobic forces. Further, peripheral proteins can usually be removed by gentle treatment of the membrane in solutions of high or low salt concentration. On the other hand, extraction of integral proteins require more drastic treatment, such as the use of detergents. Most of the plasma membranes studied exhibit numerous proteins of either type.

Early work with SPM suggested that very little protein could be removed from the membrane by gentle ionic treatment (Breckenridge et al., 1972; Smith and Loh, 1978a). However, subsequently it has been shown that a large fraction of SPM protein is in fact relatively loosely bound. Smith and Loh (1979) have reported that brief treatment of the membrane at 0°C with 0.05N NaOH could extract 65-70% of polypeptide species. They also observed that the major species of 50,000 and 40,000 molecular weight are almost completely



removed, along with many minor species in the molecular weight range of 50,000 to 90,000 thus suggesting that they are extended or peripheral in location. The major 95,000 molecular weight species, however, and minor species of higher molecular weight, are largely retained by the membrane thereby suggesting that they are integral proteins.

There have also been numerous studies of the topographical arrangement of other SPM membrane components (Hitzemann and Loh, 1975; Smith and Loh, 1976b, 1977b, 1977c). Using galactose oxidase- $\text{NaB}^3\text{H}_4$  labelling of synaptosomes, it was demonstrated that nearly all of the SPM complex carbohydrate glycoproteins and gangliosides are present on the outer surface. It should be noted, however, that all of the glycoproteins of the membrane appear to be deeply embedded in the lipid bilayer and hence some or all of them may span it. The use of pyridoxal phosphate- $\text{NaB}^3\text{H}_4$  labelling demonstrated that all of SPM's phosphatidylserine and much of its phosphatidylethanolamine are also on the outer surface. Using trypsin, it was demonstrated that the orientation of four prominent SPM enzymes -- ( $\text{Na}^+$ ,  $\text{K}^+$ ) -- ATPase, adenylate cyclase, acetylcholinesterase and alkaline phosphatase appeared to be partially or predominantly externally oriented. The opiate receptor also appears to be externally oriented.

The picture of SPM that emerges from these studies is thus one in which a large proportion of its protein

(more than 50%) is externally oriented. In addition, the amino-containing phospholipids are found largely together on one side of the membrane and are primarily externally oriented.

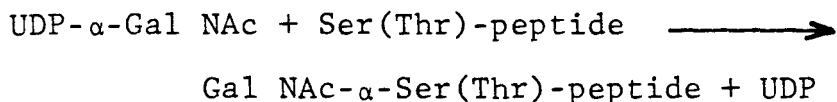
### Glycoproteins

Glycoproteins are a class of proteins that contain carbohydrate groups attached covalently to the polypeptide chain. They occur in cells both in soluble and membrane-bound forms, as well as in the intercellular matrix and in extracellular fluids. These glycoproteins are represented by many substances of considerable biologic importance and include enzymes, immunoglobulins, hormones, toxins, lectins, receptors and structural proteins. They have no unique amino acid composition, but do contain a characteristic group of sugars that frequently include D-galactose, D-mannose, D-glucose, L-fucose, D-xylose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and the various derivatives of neuraminic acid, namely, the sialic acid. The carbohydrate content of the glycoproteins may vary from less than 1% to more than 80 percent of the weight of the molecule, and as few as two to as many as seven sugar types may be present in a given protein (Spiro, 1969). The mode of linkage of the oligosaccharide side chains to the peptide backbone of glycoproteins are mainly of two types.

# 1. Linkage to the hydroxyl (OH) group of serine or threonine

In many glycoproteins which includes the submaxillary mucoprotein there is an O-glycosidic bond between N-acetyl-D-galactosamine of the side chain oligosaccharide and the hydroxyl group of a serine or threonine residue.

The initiation of synthesis of the oligosaccharide chain is catalyzed by an UDP-N-acetylgalactosamine: polypeptide- $\alpha$ -N-acetylgalactosamine transferase, which transfers N-acetyl-galactosamine (Gal NAc) to the polypeptide in an O-glycosidic linkage:



Following the incorporation of this first sugar, sialic acid (SA) may be incorporated next and requires the enzyme, CMP-sialic acid:Gal NAc-mucin  $\alpha$  2,6 sialyl transferase:



If SA is incorporated further incorporation of carbohydrate ceases. However, when other transferases are present in greater activity than sialyl transferase, the chain can be elongated by the addition of other sugars. The important transferases involved are described below.

## a. Transfer of galactose:

UDP-Gal: Gal NAc-mucin  $\beta$  1,3-galactosyl transferase



## b. Transfer of fucose:

GDP-Fuc:  $\beta$  galactoside  $\alpha$  , 1,3-fucosyl transferase (GDP-



c. Transfer of N-acetyl galactosamine:

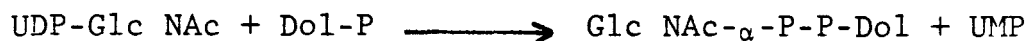
UDP-Gal NAc: mucin  $\alpha$  1,3-N-acetyl galactosaminyl transferase. This enzyme brings about the final transfer and catalyses the reaction  $\text{UDP-}\alpha\text{-Gal-NAc} + \text{Fuc-}\alpha\text{ 1,3-Gal-}\beta\text{R} \longrightarrow \text{Gal NAc-}\alpha\text{ 1,3(Fuc 1,2-)}\text{Gal-}\beta\text{-R} + \text{UDP}.$

2. Linkage to Amide Nitrogen of Asparagine

A second class of glycoproteins contain oligosaccharide side chains attached via a glycosylamine linkage between N-acetyl-D-glucosamine (Glc NAc) to the amide nitrogen of asparagine residue in the peptide backbone. The oligosaccharide side chains are two types:

- a. The "simple" or high-mannose chains which contain mainly mannose (Man) and N-acetylglucosamine residues.
- b. The "complex" chains, which in addition to mannose and Glc NAc, also contain sialic acid, galactose (Gal) and fucose (Fuc). Thus, a single glycoprotein may contain both simple and complex oligosaccharide chains; as in IgM and thyroglobulin or may contain complex (N-linked) chains as well as the O-linked chains described previously. Examples of this type are fetuin, human IgA and human erythrocyte sialoglycoprotein. The biosynthesis of these glycoproteins has unusual biosynthetic features. The carbohydrate chain of these glycoproteins has a common pentasaccharide core and is found in both the complex type and the polymannose type chains. The common core suggests a common mechanism of synthesis of the internal part of the

sugar chain and involves a lipid carrier for the core structure -- dolichol phosphate. The glycosyl lipid that serves as an acceptor for other glycosyl units is one containing a single Glc NAc residue linked to dolichol (Dol) through a pyrophosphate bridge.



The remaining sugars in the "core" oligosaccharide are then transferred to the acceptor site from their activated forms UDP-GlcNAc and GDP-Man. Lastly, the core oligosaccharide is transferred from its lipid carrier to the asparagine side chain of the protein. This is thought to take place in the rough endoplasmic reticulum. Trimming and elongation then take place in the Golgi complex and involves a series of specialized enzymes which include glucosidases, fucosidases, mannosidases and very specific glycosyl transferases.

### Glycoproteins and Neural Tissue

Studies on separated cells have shown that glycoproteins are in all of the three major cell types of brain -- neurons, astrocytes and oligodendroglia. Most of the glycoproteins of the nervous tissue are however associated with membranes and subcellular fractionation of brain has shown that they are most concentrated in synaptosomal and microsomal (axonal, dendritic and glial surface membranes) membranes. Only small amounts of glycoproteins are found in nuclear, myelin and mitochondrial fractions.

Glycoproteins are present as components of the synaptic complex. They have been located histochemically in the synaptic cleft and in association with synaptic membranes and junctional complexes (Rambourg and Le Bland, 1967; Bittiger and Schnebli, 1974; Matus and Walter, 1976) and have been identified in isolated synaptic membrane and synaptic junction fractions by direct carbohydrate analysis and reaction with lectin (Cotman and Taylor, 1974; Zanetta et al., 1975, 1977a,b; Churchill et al., 1976; Kelly and Cotman, 1977; Gurd, 1977a,b).

Glycoproteins are thought to be involved in many cell surface functions relating to synaptogenesis including cell-cell recognition, intracellular adhesion (Brunngrabner, 1969; Barondes, 1970; Roseman, 1970; Langley, 1971; Hausman and Moscona, 1975; Gombos et al., 1977; Ruffulo et al., 1978; and Morgan and Routtenberg, 1979) and the general regulation of central nervous tissue morphogenesis and neuronal and regional brain differentiation (Garber and Moscona, 1972; Pfenninger and Rees, 1976; Margolis and Margolis, 1979). Developmentally related changes in the carbohydrate composition (Di Benedetta and Cioffi, 1972; Yu and Hild, 1973; Margolis and Gomez, 1974; Ferico and DiBenedetta, 1978 and DeSilva et al., 1979) and lectin receptor activity (Zanetta et al., 1978; DeSilva, 1979) of brain and synaptic glycoproteins have been reported. In addition, age related changes in the biosynthesis of glyco-

proteins have also been reported (Dutton and Barondes, 1970; Quarles and Brady, 1971; Holian et al., 1971).

### Gangliosides

Gangliosides comprise a family of acidic glycolipids that are characterized by the presence of sialic acid. They are unusual compounds in that they contain both hydrophilic and hydrophobic regions and bear a strong negative charge. The carbohydrate portion of gangliosides is made up of molecules of sialic acid, hexoses and N-acetylated hexosamines. The hydrophobic moiety is called ceramide, and it consists of a long chain fatty acid linked through an amide bond to the nitrogen atom on carbon-2 (C-2) of the amino alcohol sphingosine. Oligosaccharides are linked through a glycosidic bond to the carbon-1 atom of the sphingosine portion of ceramide.

Gangliosides have been classified by various methods. Each laboratory has assigned symbols to denote individual gangliosides. In the earliest studies (Klenk, 1942 and Kuhn and Wiegandt, 1963), assignment of the symbol was dependent on their mobility in thin-layer chromatography (TLC) procedures. Wiegandt (1966) and other investigators have assigned symbols that bear a relation to the chemical structure. However, the most commonly used classification today is that of Svennerholm. In Svennerholm's notation, G denotes gangliosides. The capital letters M, D, T and Q refer to the

number of sialic acid residues in the molecule -- mono, di, tri, etc. Numbers 1, 2 and 3 refer respectively to gangliosides whose sum of hexose and hexosamine residues are 4, 3 and 2 respectively. Thus, GM<sub>1</sub> has one sialic acid and four hexose and hexosamine residues. The Svennerholm classification is the least complex and most easily verbalized. Table 1 shows the structures of the different classes of gangliosides.

Ganglioside biosynthesis involves a series of reactions in which sugars are transferred successively by specific glycosyl transferases from their nucleotides to the growing glycolipid acceptor. In addition, the sugar residues appear to be closely ordered and each glycosyltransferase is highly specific. In brain, the glycolipid glycosyltransferases are particulate and appear to be localized in synaptic membranes. The overall scheme of ganglioside biosynthesis has been worked out, but knowledge of the importance of the several alternate pathways possible in the stepwise build-up of the more complex gangliosides is still incomplete. It has been suggested that multiglycosyl transferase systems are involved in the entire biosynthetic reactions for each ganglioside type.

The catabolism of the entire carbohydrate chains of gangliosides proceeds by hydrolytic cleavage of the sialosyl linkages (Ohman et al., 1970) and the terminal glycosyl linkages at the nonreducing end (Gatt, 1969 and Shapiro, 1969).



### Structure of Different Gangliosides

35

These reactions are carried out by a series of glycosyl hydrolases of different substrate specificities and are thought to be localized to the lysosomes. There are several inherited progressive childhood neurological diseases in which there is a deficiency or absence of one or other of the glycosyl hydrolases involved in ganglioside catabolism, leading to excessive storage of gangliosides in neurons (Brady, 1969).

### Isolation and Purification of Gangliosides

The first step in ganglioside extraction requires extraction of the total lipids from the sample. This is usually carried out by repeated extractions of the sample with different concentrations of chloroform-methanol. Following this, there are a number of procedures available for the selective extraction of gangliosides from the total lipid extract.

#### A. 1. Procedure Based on Folch Partitioning

This procedure was first introduced by Folch et al. (1957) and is still widely used. It is based on the special property of gangliosides to partition from the organic solvent in which they are extracted into an aqueous phase. In this procedure, the lipids are extracted in chloroform-methanol and subsequently partitioned, using an aqueous 0.1 M KCl or NaCl solution. The gangliosides are then located predominantly in the upper aqueous layer,

while neutral lipids, cholesterol, phospholipids, etc. are retained in the lower organic solvent layer. However, the big disadvantage of this procedure is the contamination by phospholipids which may also be present in the upper phase along with the gangliosides. Another shortcoming of this method is the difficulty in extracting all the less polar gangliosides from the lower phase. This is a serious disadvantage when working with the numerous extraneural tissues that have a predominance of hematoside, or with brains of primates, birds and other species that have considerable amounts of sialosylgalactosyl-ceramide.

## 2. Procedure Based on DEAE-Sephadex Chromatography

The following procedure was designed to overcome the disadvantages of partitioning and to provide gangliosides in high yield and purity (Ledeen et al., 1973). In this method, the lipid extract is applied to a column of DEAE-Sephadex A-25 and neutral glycolipids, cholesterol, cerebroside and phospholipids are eluted first while gangliosides and other acidic lipids are eluted later. Different solvent systems are used for elution. The advantage of this procedure is that several hundred milligrams of a ganglioside mixture can be fractionated from a lipid extract.

## B. Base Treatment

Small amounts of contaminating phospholipids can be removed from the crude gangliosides by the mild alkaline procedure of Marinetti (1962). Ceramide linkages in sphingolipids remain intact. The alkaline methanolysis is carried out at room temperature for an hour and the mixture is then carefully acidified to a pH of 2 - 3 with methanolic hydrochloric acid. Base treatment has also been employed as a method for hydrolyzing the inner esters (Gross et al., 1977).

## C. Removal of Salt

The most common procedure used is dialysis for about 2-3 days against a large excess of cold distilled water changed 2-3 times a day. The main disadvantage of this dialysis procedure is that gangliosides must be kept above a certain concentration, called the critical micellar concentration (Kanfer and Spielvogel, 1973), during dialysis because monomeric gangliosides may escape from the dialysis bag. In addition, the procedure takes quite long and is not very convenient when dealing with a small sample size.

Another alternative procedure to dialysis, that is especially useful for eliminating certain low-molecular sugars that accompany gangliosides through the Folch-partitioning and are not removed by dialysis (Kanfer and Brady, 1967), is precipitation with trichloroacetic acid-phosphotungstic acid (Dunn, 1974). However, a serious shortcoming

of this method is that the reagent causes alteration of the tlc pattern, in which new ganglioside bands (artifacts) appear and the original bands diminish significantly (Mestrallet et al., 1976). Salts have also been removed utilizing Sephadex G-50 in an aqueous medium (Ueno et al., 1978).

Recently, Williams and McCluer (1980) have used Sep-Pak<sup>TM</sup> C<sub>18</sub> reverse phase cartridges, wherein gangliosides from upper-phase fractions can be desalted and concentrated in a short period of time and with a minimum number of steps. In addition, other lipid contaminants are also removed by this method. This method is particularly advantageous when one is dealing with small samples.

#### D. Silicic Acid Column Chromatography

A final chromatography is often needed to remove the acidic lipids like sulfatides and free fatty acids which are not destroyed by base treatment. For this purpose, chromatography on columns of unisil silicic acid, 200-325 mesh have proved very useful.

#### E. Resolution and Identification of Gangliosides

Thin-layer chromatography has become the widely used tool for the resolution of a ganglioside mixture into individual components. The most commonly used absorbents are silica gel G and silica gel HR, the former usually

giving better separations due to the presence of calcium in the binder. Different solvent systems have been used with good success (Penick et al., 1966; Marinetti, 1967; Colowick and Kaplan, 1969; Coleman and Yates, 1978 and Zanetta et al., 1980). Gangliosides are visualized by spraying the plates with resorcinol reagent of Svennerholm (1957) and the quantification of the sialic acid in each ganglioside band separated by thin-layer chromatography can be carried out by the method of Suzuki (1965).

Separation and quantitation of gangliosides have also been done by gas chromatography (Sweeley and Vance, 1967), by high-pressure liquid chromatography (Bremer et al., 1979) and also fluorometrically (Warren, 1959 and Aminoff, 1959).

Alternate procedures for the isolation of gangliosides from small tissue samples have also been used. Irwin and Irwin (1979) have reported a simple rapid method for ganglioside isolation from small amounts of tissue using silicic acid columns. Similarly, Harth et al. (1978) have reported a procedure for the separation of gangliosides from a total lipid extract by thin-layer chromatography using three successive solvent systems.

### Localization of Gangliosides in Neural Tissue

The concentration of ganglioside sialic acid in brain has been shown to be over ten times higher than that in any other tissue (Long and Staples, 1959; Puro et al.,

1969). Furthermore, the percentage of the total sialic acid that is lipid-bound is much higher in brain than in any other tissue. Over half of the sialic acid in brain tissue is bound to lipid; while six percent or less is lipid-bound in lung, kidney, liver and other non-neural tissues.

Early in the development of ganglioside biochemistry, it was been shown (Klenk and Langerbeins, 1941; Svennerholm, 1957) that gray matter had a substantially higher concentration of gangliosides than white matter. Various studies have revealed small but significant differences in total ganglioside concentration and pattern among different gray matter regions and also in different white matter tracts (Lowden and Wolfe, 1964; Suzuki, 1967; Dominick and Gielen, 1968). Cerebellum, for example, differs from the cerebral cortex in having an unusually high level of trisialoganglioside ( $GT_{1b}$ ) and a greatly reduced monosialoganglioside ( $GM_1$ ). The latter is found in high proportions in white matter regions due to its predominance in myelin (Suzuki et al., 1967; Ledeen et al., 1973). Various components of the optic system were found to differ significantly from the CNS in general (Ledeen and Yu, 1976b). Retina, for example, has one eighth the ganglioside concentration of gray matter, and  $GD_3$  is the major type in the case of mammals (Handa and Burton, 1969; Holm et al., 1972; Edel-Harth et al., 1973). Developmental changes and species differences have also

been noted (Dreyfus et al., 1975).

In recent years, much attention has been focused on the distribution among cellular elements of the brain and several laboratories have now analyzed the gangliosides of isolated neuronal and glial cells. Brain gangliosides have been found to be located mainly in the neurons. The sub-cellular fractions of brain having the highest ganglioside content are listed below:

- a. the subfraction containing nerve endings and nerve ending membranes -- the synaptosomal fraction (Yohe et al., 1980)
- b. the microsomal fraction which contains endoplasmic reticulum, as well as axonal, dendritic and glial surface membranes (Wolfe, 1961; Weigandt, 1967; Lapetina, 1967)
- c. the myelin fraction (Suzuki et al., 1967, 1968).

Wolfe (1961) found that the microsomal fraction from rat brain contained the highest concentration of gangliosidic sialic acid per milligram (mg) of protein, but 40 percent of the total gangliosidic sialic acid of the original homogenate was recovered in the synaptosomal fraction. The myelin-enriched fraction contained 24 percent and the mitochondria only 7 percent. Gangliosides were virtually absent in the cytosol, although it was necessary to centrifuge the homogenate for a long period of time (over two hours at 100,000 g) to obtain a supernatant free of small ganglioside enriched particles. The association of gangliosides



with synaptosomes, microsomes and myelin have been confirmed in a number of other studies (Svennerholm, 1964; Wiegandt, 1967; Lapetina et al., 1967; Brunngraber et al., 1967; Suzuki et al., 1967, 1968). Within the synaptosomes, the synaptic plasma membrane undoubtedly contains the highest concentration of gangliosides (Breckenridge et al., 1972; Aurora et al., 1973).

Recently, Seyfried et al. (1982) have studied the cellular localization of gangliosides in the central nervous system. Using a series of mouse mutations (Weaver, Stagger and Lurcher mutations) which result in selective destruction of specific populations of neurons in the cerebellum, they were able to demonstrate an enrichment of specific gangliosides in particular cell types. They reported that GD<sub>1a</sub> appeared to be more heavily concentrated in granule cells while GT<sub>1a</sub> was mainly concentrated in Purkinje cells. In addition, they found that GD<sub>3</sub> was enriched in reactive glial cells, suggesting that it may play an important role during the morphological transformation of neural membranes.

### Developmental Changes of Gangliosides

The ganglioside concentration changes during development. There is a sharp increase of gangliosides in brain during the embryological and fetal stages of development. It has been observed that there is a 20-fold increase from the 5th day to hatching in chick (Garrigan and Chargaff,

1963), a two-fold increase from the end of the third fetal month to the time of delivery in humans (Svennerholm, 1964) and a three-fold increase from the eighth day before birth to birth in the rat (Tettamanti, 1971).

After birth, a further increase of the ganglioside content occurs in the brain. In human brain, Suzuki (1965) has shown a two-fold increase from birth to the adulthood. In rat brain, the maximum level is reached at the 18th-20th day with the increase having a slow rate for the first 6-8 days followed by a more rapid rate till the 12th-14th day (Suzuki, 1965). The adult level is about three times higher than that at birth, although a much higher rise (about 50-fold) has been reported by Rosenberg and Stern (1966).

This high accumulation of gangliosides in rat brain (from birth to 20-25th day) is due to the formation of myelin which contains gangliosides, the formation of new synaptic complexes which are very rich in gangliosides, and the formation of ganglioside rich membranous structures, preparatively included in the microsomal fraction (possibly dendrites in rapid growth and branching).

The ganglioside pattern also changes considerably during development. In the rat at birth, the ganglioside pattern is characterized by the presence of four major gangliosides --  $GM_1$ ,  $GD_{1a}$ ,  $GD_{1b}$  and  $GT_{1b}$ , followed by traces of  $GM_2$  and  $GM_3$ . After birth, in the period of the accumulation of gangliosides, the most striking event is

the drastic increase of  $GD_{1a}$  (Suzuki, 1965) and therefore its percentage of the total ganglioside content goes up sharply. After the 20th day of age until adulthood  $GD_{1a}$  decreases,  $GM_1$  remains unchanged, while  $GD_{1b}$  and  $GT_{1b}$  undergo a slow but constant increase. The maximum level of  $GQ_1$  is reached at the 15th day, followed by a decrease until the very low level of the adult animal (Tettamanti, 1971). In newborn human brain, Svennerholm (1963) found a ganglioside pattern characterized by a very large  $GD_{1a}$  fraction, followed by  $GM_1$ , with small amounts of  $GD_{1b}$  and  $GT_{1b}$ . After birth, according to Suzuki (1965), the level of  $GM_1$  remains unaffected while  $GD_{1b}$  and  $GT_{1b}$  increase markedly, in parallel with diminution of  $GD_{1a}$ . In fetal human brain, the pattern is reported to be closer to that of the adult (Svennerholm, 1964).

Thus, the main features of the developmental changes of the ganglioside pattern in mammalian brain can be outlined as follows.

- a.  $GM_3$ ,  $GM_2$ ,  $GM_1$  and  $GD_{1a}$  appear at a very early stage. Among them,  $GD_{1a}$  undergoes a very rapid increase (the maximum level being reached around birth in human and at the 18th-20th day after birth in rat), followed by a drastic decrease.  $GM_1$  inversely follows the pattern of  $GD_{1a}$ . While  $GM_3$  and  $GM_2$  rapidly decrease and reach very early (before birth) the final, low level.
- b.  $GD_{1b}$  and  $GT_{1b}$ , which probably appear at a later stage,

undergo a gradual increase until the adult stage.

c. GQ<sub>1</sub> has the latest appearance.

### Function of Gangliosides

The localization of gangliosides in the plasma membrane of nerve terminals suggests that these glycolipids may be involved in ion fluxes associated with action potentials (McIlwain, 1959, 1961). They may also function as receptors for neurotransmitters (Wooley and Gommi, 1965). There is currently considerable evidence that these glycolipids are involved in the release of neurotransmitters at the nerve terminals and are involved in receptor activity for tetanus toxin (Helting et al., 1977), cholera toxin (Gill, 1977), Sendai viruses (Fishman and Brady, 1976), and for peptide hormones (Lee et al., 1976 and Meldolesi et al., 1976). Gangliosides have also been implicated in cell-cell recognition (Irwin, 1974) and at least four enzymopathies are known to involve ganglioside metabolism (Adachi et al., 1978). Finally, gangliosides have long been suspected to play a role in brain cell differentiation, recognition and synaptogenesis (Wiegandt, 1968; Irwin, 1974; Marchase, 1977).

### Fetal Alcohol Syndrome

It has been only in the last few years that a pattern of malformation in children born to women with severe chronic

alcoholism has been identified and termed the "fetal alcohol syndrome" (FAS) or "fetal alcohol effect" (FAE).

Since the initial discovery of this disorder, historical evidence has been brought to light indicating that an association between maternal alcoholism and serious problems in the offspring was not a new observation. Evidence is even available from classical Greek and Roman mythology suggesting that maternal alcoholism at the time of conception could lead to serious problems in fetal development. This led to an ancient Carthaginian ritual forbidding the drinking of wine by the bridal couple on their wedding night in order that defective children might not be conceived (Haggard and Jellinek, 1942). Even in the early 1900's there has been documentation of an increased frequency of early fetal death and early infant mortality, as well as decreased weight of surviving children born to chronic alcoholic mothers (Ladraque, 1901; Roe, 1944; Leconte, 1950; and Christiaens et al., 1960).

In 1968, Lemoine et al. (1968) described a pattern of prenatal and postnatal growth deficiency, developmental delay, mental retardation, microcephaly, and a number of facial, limb and cardiac defects, and attributed these defects to the effects of alcohol in utero. These observations were neglected until 1973, when Jones et al. (1973) described the same pattern of abnormalities and termed it the Fetal Alcohol Syndrome. The close similarities between the reports described independently by two research groups in different

parts of the world, and later by others as well, suggest strongly the validity of the observations.

The frequency of severe chronic alcoholism in the United States has been estimated at 1 in 1000 pregnancies (Hanson et al., 1976). It has also been estimated that 30 to 50 percent of such pregnancies will show the FAS signs (Hanson et al., 1976). There is a 17% mortality rate associated with this syndrome (Jones et al., 1974; Hanson et al., 1976). Most FAS offspring are classified as small for date (Ulleland, 1972; Jones and Smith, 1973; Jones et al., 1973, 1974a, 1974b; Palmer et al., 1974; Saule, 1974; Root et al., 1975; and Hanson et al., 1976) and exhibit craniofacial, skeletal, cardiac and genital abnormalities (Jones and Smith, 1973; Jones et al., 1973, 1974b; Palmer et al., 1974; Saule, 1974; Root et al., 1975; Hanson et al., 1976; Mulvihill et al., 1976; Mulvihill and Yeager, 1976). Nearly half of the FAS neonates are mentally deficient (Jones et al., 1974; Palmer et al., 1974; Hanson et al., 1976; Mulvihill et al., 1976; Mulvihill and Yeager, 1976; Streissguth, 1976; Streissguth et al., 1978; and Streissguth et al., 1981) and have altered EEG patterns (Root et al., 1975; Havlicek and Childaeva, 1976). In addition, it has been shown that intrauterine exposure to large quantities of ethanol can produce structural abnormalities in the brain regardless of whether external features of the syndrome are present (Clarren et al., 1978). These and numerous

other physical abnormalities occur in the children of alcoholic mothers who were given nutritional and vitamin supplements during their pregnancy (Hanson et al., 1976 and Mulvihill et al., 1976). Evidence that alcohol or its metabolite is the prime factor in the abnormalities seen in the Fetal Alcohol Syndrome is substantiated by the fact that a mother can decrease the likelihood of having a child with Fetal Alcohol abnormalities if she decreases her alcohol consumption during pregnancy (Ouelette and Rosett, 1977).

#### Human Studies

There are presently three major prospective studies in the United States which are investigating the relationship among fetal and infant anomalies, amount of alcohol consumed by the mother, and other risk factors that are shared by these mothers such as nutritional deficiency, heavy smoking, use of other drugs, emotional stress and traumatic injuries.

The Boston group has studied 633 women, of which 9% were classified as heavy drinkers -- women who consumed 5 or more drinks on occasion with a minimum daily average of 1.5 drinks. Women who drank less than once a month were classified as abstinent or rare drinkers and the remaining were classified as moderate drinkers. A study of their diets showed that they were not significantly different from the diets of the total clinic population. Detailed

pediatric, neurological and developmental examinations on the 322 newborn infants revealed that the frequency of all abnormalities was twice as great in offspring of heavy drinkers when compared with the frequency in the other two groups. The abnormalities included congenital malformations, growth disturbances and functional abnormalities (Ouellette et al., 1977).

A second longitudinal prospective study on alcohol and pregnancy by the Seattle group involves 1529 predominantly white, middle-class pregnant women and their offspring. In addition to alcohol, caffeine and smoking, other drug intake and diet were evaluated. Different subsamples from the group of 500 newborn infants were selected for follow-up at birth, 8 and 18 months of age. Half of these were from the heaviest drinkers while the remaining half were from the abstainers and light drinkers. Examinations have revealed that more offspring born to mothers with the higher level of alcohol consumption had clinical features of altered growth and morphogenesis as compared to offspring of mothers with a lower level of alcohol intake. In addition, significantly lower mental and motor development and lower length and weight were found on follow-up of these infants at the age of 8 months (Streissguth et al., 1981).

The third and largest prospective study is being conducted at Loma Linda University and preliminary descriptive findings on 5878 women attending the prenatal clinics



are available (Kuzma and Phillips, 1977).

Clarren et al. (1978) have recently demonstrated structural alterations in the brains of infants exposed to alcohol in utero. Four brains showed similar malformations related to failure or interruption of neuronal and glial migrations. While the type of malformation was similar in each case, the location of the malformations varied from subject to subject. The most consistent anomalies were cerebellar dysplasias and heterotopic cell cluster, especially on the brain surface. In one case, the malformations were primarily in the cerebrum and there was associated microcephaly. Subtentorial anomalies produced hydrocephalus in two cases, but had no outward effect on head size in another patient. Similar malformations have been described by Majewski and Bierich (1979) in one patient.

### Animal Models

Numerous animal models have been used in an attempt to induce alcoholism in laboratory animals. Most of these models can be classified on the basis of route of administration, chronicity of exposure, and purpose of the model. Models that provide continuous exposure to alcohol are most frequently used and are generally selected for studies of physical dependence, tolerance and chronic toxicity and are known as the 'chronic models.' The second category or

'reinforcement models' are designed to evaluate the psychological effects of alcohol, especially its role as a behavioral reinforcer. Finally, the third group or 'genetic models' are developed from certain genetic strains or breeding lines that have been established to maximize specific physiological responses to alcohol. Studies with such animals can provide important information about the genetic and biochemical substrates of many alcohol effects.

### Chronic Models

- a. Intubation Models: Injection or intubation of alcohol solutions is a direct method of drug administration. Several researchers (Branchey et al., 1971; Ellis and Pick, 1972; Leblanc et al., 1975) have used this procedure to induce alcohol tolerance and physical dependence or to study a range of alcohol effects (Ellis and Pick, 1976, 1980). Direct administration methods require frequent handling of the animals which is often highly stressful. In addition, if tolerance and dependence is induced too rapidly, the animals become incapacitated and malnourished.
- b. Inhalation Chambers: This procedure was developed by Goldstein and Pal (1971), and alcohol is administered as a vapor by establishing an alcohol atmosphere in a small chamber. Alcohol is absorbed from the alveolar spaces of the lung and since alveolar concentration is stable as long as the chamber atmosphere is kept constant there is little

fluctuation in blood alcohol concentrations.

c.     Subcutaneous Implants: This novel technique for continuous alcohol exposure was developed independently by two groups (Erickson et al., 1978; Ellison, 1978) and makes use of subcutaneous semipermeable implants for a slow, continuous diffusion of alcohol. The main drawback of this procedure is the surgical implantation and also the frequent handling of the animals in order to replenish the reservoir solution.

d.     Schedule Induced Polydipsia: This method was proposed by Falk et al. (1976) to induce animals to consume large quantities of alcoholic solutions and capitalizes on the instinctive tendency of rats to drink water after consuming any food. This procedure creates high food consumption rates with operant procedures and provides alcoholic solutions in place of water. However, this procedure is somewhat controversial due to the large fluid volumes that the rats consume.

e.     Liquid Diets: This most widely used chronic model provides alcohol as a component of a liquid diet intended to provide the animal's total nutritional needs. The liquid diet model has the advantage that it provides sufficient intake of key nutrients to meet the animal's needs, allows accurate measurement of consumption and permits the investigator to control alcohol intake, and hence is a method of choice for studies on the Fetal Alcohol Syndrome.

This diet was used by Lieber and DeCarli (1973,1974) and Lieber et al. (1975) in studies of alcohol induced liver pathology and has been developed for rodents, macaques and baboons. The main advantage of the Lieber-DeCarli liquid diet is the assurance of replicability of the ingredients and formula, complete control of the composition, and the lack of stress on the animals due to surgical and handling procedures. Unfortunately, it is not free of drawbacks. The primary disadvantage is that the diet tends not to be very palatable to the animals and is calorically dilute. In addition, the diet is formulated to provide the minimal nutritional needs of a normal animal during alcohol consumption and hence it is not clear whether those needs are met during certain periods of an animal's life such as pregnancy. However, Weiner (1980) has proposed a higher protein modification of the basic diet to meet the higher demands of pregnant animals in Fetal Alcohol Syndrome studies. Despite the mentioned drawbacks, the liquid diet procedure is widely used and has generally been accepted as a reliable method for providing chronic alcohol exposure. With careful management and well-designed controls, the liquid diet has proved to be one of the most useful and efficient alcohol models for alcohol research.

f. Reinforcement Models: These represent a group of models called 'preference models' and have been used frequently to study alcohol reinforcement (Myers and Holman,

1971). In this procedure, experimental animals are presented with two or more bottles of solutions, one containing only vehicle and the other containing vehicle plus alcohol.

Significantly more consumption from any bottle is considered an indication of the animal's preference for the solution in the bottle. Preferential drinking of a drug solution is thought to indicate that the contents of that bottle are reinforcing.

g. Genetic Models: These models consist of several lines of animals from various species that have been selectively inbred to increase the predominance of specific responses to alcohol and have recently been reviewed in detail by Eriksson (1980).

#### Animal Studies of the Fetal Alcohol Syndrome

Recent years have witnessed a proliferation of investigations or studies of the toxic effects of ethanol on prenatal development in laboratory animals, using some of the routes for the administration of alcohol that have been described previously. These investigations include numerous biochemical, behavioral, as well as neuroanatomical and neurochemical studies

A number of investigators (Sandor and Amels, 1971; Skosyreva, 1973; Tze and Lee, 1975; Kornick, 1976; Randall et al., 1977; Abel, 1978 and Sulik et al., 1981) using different models for the administration of ethanol have reported increased resorption rates, decreased fetal and neonatal

birth weights as well as a number of externally visible facial, skeletal, heart, abdominal and urogenital malformations (similar to those observed in humans) in the embryos, fetuses and offspring of mothers exposed to alcohol.

In addition, many of the studies have reported that the severity of craniofacial and other abnormalities is greater with larger doses of ethanol exposure during gestation or embryogenesis.

Many researchers have also been investigating the behavioral effects of intrauterine exposure to alcohol. Yanai and Ginsburg (1976) have shown that the offspring of two inbred strains of mice (C57 and DBA) that were fed alcohol were more susceptible to audiogenic seizures and exhibited a shorter latency to seizure. They also noted hyperactivity and slow maturation of the righting reflex in pups born to mothers which received alcohol during the last two weeks of pregnancy than in either pair-fed or normally-fed control mice. In another study, they found that ethanol administered transplacentally significantly increased the cricket-predation behavior of DBA/1BG offspring, but not of the offspring of the less predatory C57/10BG strain.

Elis et al. (1976) reported that ethanol given to pregnant mice throughout gestation resulted in more aggressive male offspring with lower concentrations of 5-hydroxytryptamine in the brain.

Studies performed on rat models have also shown similar results. Bond and DiGiusto (1976, 1977a, 1977b) have shown that prenatal ethanol exposure leads to an increase in open-field activity of offspring prior to puberty and that these animals display shock-avoidance learning deficits when they are tested as adults.

Increased spontaneous locomotor activity has also been reported by Branchey and Friedhoff (1976) in rats that were treated prenatally with alcohol. Hyperactivity has been the most commonly reported behavioral effect, however, deficits have also been reported in two-way (Abel, 1979, 1980) and passive avoidance (Lochry and Riley, 1979) as well as an increased resistance to extinction (Riley et al., 1980).

There have also been a number of neuroanatomical studies done on animals exposed to alcohol both prenatally and postnatally.

Bauer-Moffett and Altman (1975, 1977) found that ethanol vapor inhalation between postnatal days 3 and 20 produced a severe reduction in the brain weight of rats and that the cerebellar weight loss was twice that for the other brain regions. They observed that all the layers of the cerebellar cortex (medullary, granular and molecular) were reduced in thickness. Quantitative assessment of two major neuronal populations of the cerebellar cortex -- the large, prenatally-formed Purkinje cells and the small,

postnatally formed granule cells -- showed a reduction of both cell types in all lobules of the cerebellum for the ethanol treated animals. Using autoradiographic techniques in their studies, they showed that the pattern of granule cell neurogenesis was essentially the same in ethanol and control rats. The authors hypothesized that the granule cell loss was due either to a direct effect on germinal cells or to an indirect transneuronal effect by way of the Purkinje cells.

Anderson and Sides (1979), using a similar method but with a higher dosage and from 24 hours after birth, confirmed the reduction of cell numbers in the internal granular layer. They observed that the distribution of cells in this layer was also abnormal, the usual pattern being absent.

Riley and Walker (1978) and Walker et al. (1980) studied the effects of long term ethanol consumption in mice and rats and observed that it resulted in a fifty to sixty percent loss of dendritic spines on hippocampal pyramidal cells and dentate gyrus granule cells as determined by quantitative analysis of Golgi-impregnated materials.

Barnes and Walker (1981) studied the effect of prenatal ethanol exposure in the offspring of rats that consumed 15.5 g/kg of ethanol per day on days 11-21 of gestation, on the development of the dorsal hippocampal pyramidal and dentate gyrus granule cells. They observed a 20% reduction



in dorsal hippocampal pyramidal cells and no reduction in the number of dentate gyrus granule cells. This permanent reduction in the number of prenatally formed hippocampal neurons was observed in animals that exhibited no external dysmorphology.

West et al. (1981) also reported evidence of aberrant hippocampal development in rats that otherwise appeared normal. They observed a dramatic alteration in the topographical organization of the hippocampal mossy fiber system of the ventral hippocampus in offspring of rats that ingested 12.2 g/kg of ethanol during days 1 to 21 of gestation. The ethanol exposed rats showed a conspicuous band of distal infrapyramidal mossy fibers in hippocampal subfield CA3 at middle and more temporal levels of the hippocampus. These aberrant fibers appeared to be a continuation of the rostral (normal) distal infrapyramidal band.

Sherwin et al. (1981) studied the effects of ethanol in the offspring of rats that consumed a semipurified liquid diet in which ethanol accounted for 40% of the total caloric intake. Histological analysis, both qualitative and quantitative, demonstrated a decrease in dendritic spine counts in the pyramidal CA-1 cells of the hippocampus which is similar to the findings of Riley et al. (1978) for the adult mouse. They also observed much less branching and shorter dendrites and suggest that this in utero effect of alcohol produces a retardation in the development of the

neurons.

Kornguth et al. (1979) gave a liquid diet containing ethanol to rats throughout gestation and performed histological and biochemical analyses on the cerebellums and cerebrums in the offspring at 11 and 14 days postnatally. They observed that pups which were exposed to ethanol in utero had significantly smaller body weights, cerebrums and cerebellums than pair-fed controls. In addition, the cerebellar mass was reduced by 10% and the cerebral weight by 3%. Histological analyses revealed that the cerebellar external granule cell layer of ethanol pups was significantly thicker at 11 and 14 days postnatally. Cerebellar ornithine decarboxylase activity (which is an indicator of rapid cell division) was also higher at day 11 in ethanol treated pups. These observations suggest that there is a retarded migration from the external granular layer and a prolonged proliferative activity in the layer which normally starts to involute at day 11.

Volk (1977) observed a delayed cerebellar maturation in light microscopic investigations in the offspring of rats that were given alcohol in their daily drinking water during pregnancy. In subsequent studies, Volk et al. (1981) observed an impairment of maturation of the Purkinje cells of the cerebella in the offspring of rats that were exposed to ethanol by means of a vitamin supplemented liquid diet prior to and throughout gestation. The Purkinje cell nuclei of

ethanol pups were significantly smaller and also there was a delayed cytoplasmic maturation of the Purkinje cells which mainly involved the rough endoplasmic reticulum.

Hammer and Scheibel (1981) studied qualitatively the deep neocortical pyramidal cell dendritic structure by golgi staining techniques in neonatal rats whose mothers were exposed to 5% ethanol in a protein enriched liquid diet. They observed that in the ethanol exposed pups dendritic extent and branching of the pyramidal neurons were much less. Somata were also much smaller and rounder than control neurons. These characteristics of neuronal structure suggest that fetal alcohol exposure causes a delay of neuronal development.

A number of biochemical studies has been reported on the effects of alcohol on fetuses and offspring of rats exposed to ethanol during pregnancy.

Henderson and Schenker (1977) reported slightly elevated protein concentrations in the brains of alcohol exposed pups, but heart, liver and kidney showed no change. DNA synthesis rates were normal, but the total RNA levels were significantly depressed by about 10 - 30% in all the organs studied.

In related studies, Rawat (1975) found an impairment in  $^{14}\text{C}$ -leucine incorporation into cerebral ribosomes and a decrease in brain transfer RNA, total RNA and DNA in both fetal as well as newborn offspring of rats exposed to

alcohol. This inhibitory effect of ethanol on protein synthesis was found to be concentration-dependent. Rawat (1976) also found decreases in total hepatic RNA content, hepatic RNA/DNA ratio, and ribosomal protein content of the fetal livers of rats exposed to alcohol in utero.

Sze et al. (1976) reported an increased alcohol dehydrogenase activity as well as an increase in hepatic microsomal mixed function oxidase activity in mice that had been exposed to ethanol in utero.

Chronic ethanol consumption by pregnant rats caused alterations in the developmental pattern of ornithine decarboxylase activity in the heart and brain of offspring, thus suggesting that ethanol interferes with fetal polyamine metabolism (Thadani et al., 1977b, 1977c).

Druse and Hofteig (1977) reported that the consumption of alcohol one month prior to conception and throughout gestation resulted in a premature onset and slow-down of active myelination. The ethanol exposed pups showed an elevated synthesis of myelin, particularly the heavy or immature myelin at 18 and 25 days and a decreased synthesis of all the myelin subfractions -- heavy, medium and light at 54 days. Developing ethanol exposed pups also showed abnormal rates of incorporation of [ $^3\text{H}$ ]leucine and [ $^{14}\text{C}$ ]glucose into the myelin subfractions. At 18 and 25 days the ethanol pups incorporated more of both precursors into all fractions of myelin, while at 54 days there was a decreased incorporation.

Although the quantity and rate of synthesis of individual myelin subfractions were abnormal in ethanol pups, the protein composition of the various subfractions was normal.

In contrast, the offspring of female rats fed an ethanol liquid diet only during the last two weeks of gestation had a near normal pattern of myelination (Hofteig and Druse, 1978). This suggests that the myelin abnormalities observed in the chronic study may be due to the metabolic or hormonal effects of chronic alcohol consumption rather than the teratogenic action of ethanol or its metabolite.

There have been a number of studies on the steady-state levels of catecholamines (See Druse, 1981 for a review of the topic) in the fetuses and offspring of rats exposed to ethanol prior to and during various periods of gestation. Maternal ethanol consumption resulted in decreased adrenal levels of catecholamines (Lau et al., 1976), decreased hepatic levels of dopamine (Rawat and Kumar, 1977) and decreased whole brain and hypothalamic levels of norepinephrine (Detering et al., 1980). Detering et al. (1981) have reported that the deficit of norepinephrine in the hypothalamus of pups exposed to ethanol is not a transient deficit, but rather a long-lasting one. However, normal whole brain levels of dopamine and norepinephrine have been reported in the offspring of mice (Elis et al., 1976, 1978; Krisiak et al., 1977) and rats (Rawat, 1977) that had been exposed to ethanol during gestation. Differences between

these studies and those reported by Detering et al. (1980, 1981) may be due to differences in either the route, dose or timing of ethanol administration or may be due to brain regional effects.

Normal brain steady-state levels of serotonin have been reported in the offspring of rats (Rawat, 1977) and mice (Boggan et al., 1979) that were fed ethanol liquid diets at various times of gestation. Maternal ethanol consumption however resulted in increased levels of glutamate and  $\gamma$ -aminobutyric acid (GABA) but decreased levels of acetylcholine in fetal brains (Rawat, 1977).

Thadani et al. (1977a) studied the synaptosomal uptake of [ $^3$ H]tyramine and the conversion of tyramine to octopamine. They found that the uptake of tyramine and synthesis of octopamine were increased in rats whose mothers were given an ethanol liquid diet from either day 14 or 18 of gestation through parturition. This effect persisted until the 24th day of age. Ethanol appeared to shift the development of the noradrenergic synapses to an earlier age period.

Slotkin et al. (1980) studied synaptosomal uptake of norepinephrine, dopamine and serotonin and used it as an indicator of synaptic development, as the procedure provides an estimate of the numbers of the synaptic terminals for each neurotransmitter. They found that ethanol exposure during the perinatal period did not produce general retardation

of synaptogenesis of catecholaminergic systems. However, uptake of serotonin displayed a small but consistent deficit in ethanol exposed pups indicating a possible retardation in serotonergic synaptogenesis.

Finally, ethanol also exerts a variety of toxic effects on reproduction in eutherian mammals. Badr and Badr (1975) found that alcohol is mutagenic in the mouse dominant lethal test and that it exerts the most marked effects against epididymal spermatozoa and late spermatids. Khvatov (1975) found that ethanol affects fertilization and early embryonic development in the golden hamster. In this species, the dynamics of the ova's passage along the tube after ovulation is modified by ethanol, so that normal fertilization is disturbed and polyspermia and zygote death follow.

### Rationale of the Proposed Studies

From the numerous studies cited above it is evident that the fetal alcohol syndrome is a well documented disorder and is characterized by the presence of varying degrees of mental retardation and altered EEG patterns. Furthermore, CNS dysfunction has been reported in offspring of alcoholic mothers even when the external criteria (cranio-facial, skeletal and growth abnormalities) of FAS are not evident, suggesting that it may be the most sensitive manifestation of an in utero ethanol exposure. Neither the range of

neuronal defects nor the mechanism influencing these alterations during development are understood.

Results from human studies indicate that the widespread lesions within the CNS are probably the result of glial and neuronal migratory failure (Clarren et al., 1978). A variety of animal studies suggest both delay in brain development (Thadani et al., 1977; Sherwin et al., 1980) as well as permanent CNS defects (Slotkin et al., 1980; Sherwin et al., 1981; Barnes and Walker, 1981; West et al., 1981 and Hammer and Scheibel, 1981). Previous work from this laboratory (Druse and Hofteig, 1977 and Hofteig and Druse, 1978) suggest that the CNS abnormalities may be related to alterations in CNS membranes.

In addition, several of the neurochemical studies of the fetal alcohol syndrome suggest that the synapse is affected in particular in the ethanol offspring in terms of steady-state levels, synthesis, uptake and release of neurotransmitters (Rawat, 1977; Thadani et al., 1977; Slotkin et al., 1980; Druse, 1981). In spite of the potential seriousness posed by prenatal ethanol exposure on the CNS and particularly on the synapse there is a paucity of information concerning the effects on the synaptic membrane.

The purpose of this dissertation then, is to gain an understanding of the neurochemical alterations that might be responsible for CNS disturbances frequently



associated with this syndrome by examining the synthesis of glycoproteins and gangliosides in the synaptic plasma membrane as well as other CNS membrane fractions. This will be accomplished by determining if there are any differences in the ethanol offspring by examining the distribution of radioactive precursors such as fucose in the case of glycoproteins and sialic acid in the case of gangliosides in the synaptic membrane fraction. These studies are conducted at 10, 17, 24 or 31 days of age. These age points were selected in order to reflect the early, middle and later periods of active (rat) synaptogenesis (Jacobson, 1978). Aghajanian and Bloom (1967) have reported a rapid increase in the number of synaptic junctions in the rat parietal cortex between the 14th and 26th postnatal days. Eayrs and Goodhead (1959) have estimated a ten-fold increase in synaptic connectivity between days 12 and 30 after birth in the cerebral cortex of the rat. Kelly and Cotman (1981) have reported that the rate of increase in synaptic plasma membrane protein content displayed an apparent linear phase between days 10 to 20. By day 20, the SPM protein content began to plateau, attaining greater than 95% of adult values by day 25. These results correlate very well with the appearance of junctional complexes in situ (Aghajanian and Bloom, 1967). In addition, the research reported in this dissertation will include the effects of alcohol on synaptic

membrane glycoproteins in the offspring that were cross-fostered to normal chow-fed mothers. These studies are important because cross-fostering is believed to avoid potential nutritional and nurturing problems and is also believed to eliminate problems of maintaining ethanol offspring with mothers who may have alcohol- or withdrawal-related hormonal and metabolic disturbances. The hypothesis that the metabolic state of the mother could be responsible for some of the effects of alcohol seen in the offspring will be examined in this dissertation by the inclusion of a reverse cross-fostered study.

The carbohydrate portion of gangliosides and glycoproteins are known to be cell-surface structural elements, involved in many cell surface functions relating to synaptogenesis, including cell-cell recognition, synaptic connectivity, intracellular adhesion (Brunngraber, 1969; Barondes, 1970; Roseman, 1970; Langley, 1971; Damstra-Entingh et al., 1974; Hausman and Moscona, 1975; Gombos et al., 1977; Ruffulo, et al., 1978 and Morgan and Routtenberg, 1979) as well as the general regulation of central nervous tissue morphogenesis and neuronal and regional brain differentiation (Garber and Moscona, 1972; Pfenninger and Rees, 1976; Margolis and Margolis, 1979). In addition, glycoproteins function as structural proteins and enzymes (Spiro, 1969) and gangliosides have been shown to function as receptors for neurotransmitters (Wooley and Gommi, 1966). Thus, an

alteration in the content or structure of the synaptic plasma membrane glycoproteins or gangliosides could have serious pathological and functional consequences. Changes in the content, synthesis or structure of glycoproteins could result in altered synaptic connectivity (Damstra-Entingh, 1974) and possibly neural dysfunction.

Alterations of the carbohydrate structure of cell-surface gangliosides (as in virally-transformed cells) have been shown to cause increased cellular mobility, loss of growth control and recognition and a high saturation density in tissue culture (Brady et al., 1973). Hence, abnormal ganglioside synthesis due to an in utero exposure of ethanol could indicate neuronal abnormalities.

There are several different types of studies (neuro-anatomical, neurochemical and behavioral) that suggest that certain brain regions are more susceptible to the damaging effects of in utero ethanol exposure than other areas. More specifically, neuroanatomical studies suggest that the hippocampus, cerebellum and cerebellar cortex are particularly susceptible to the effects of ethanol exposure in utero. Barnes and Walker (1981) reported a 20% decrease in dorsal hippocampal pyramidal cells, while West et al. (1981) found abnormalities in the topographical organization of the hippocampal mossy fiber system which represents the major excitatory pathway connecting the dentate gyrus with the CA<sub>3</sub> hippocampal field. Kornguth et al. (1979)

have reported a decrease in cerebellar mass and weight; while Hammer and Scheibel (1981) found that pyramidal cells have decreased dendritic branchings. In addition, there was delayed maturation of Purkinje cells in the cerebellar cortex of ethanol-treated offspring.

Neurochemical evidence of regional effects of in utero exposure to ethanol comes from studies that demonstrated altered steady-state levels of norepinephrine in the hypothalamus (Detering et al., 1981) and from studies that found increased content of gangliosides in the hippocampus and brain stem of ethanol pups (Druse, Oden and Haas, unpublished observations). Behavioral studies also indicate that the hippocampus is one brain region that is most affected by prenatal exposure to ethanol. Hyperactivity has been the most commonly reported behavioral effect (Randall and Riley, 1981) but deficits have also been reported in two-way (Abel, 1979; 1980; Bond, 1980) and passive avoidance (Riley et al., 1979; Lochry and Riley, 1980) as well as an increased resistance to extinction (Riley et al., 1980). It should be pointed out that such deficits in response are consistently found in animals with hippocampal damage (Kimble, 1975).

In view of the cited studies of animal models of FAS that emphasize the importance of performing brain regional analyses when possible, a part of this

dissertation will also examine the influence of maternal ethanol consumption on the development of gangliosides of discrete brain regions in offspring. It has been shown that accumulation of gangliosides in the cerebellum takes place between 8 - 28 days (Vincendon et al., 1975; Gombos et al., 1980 and Vrbaski, 1980); between 8 - 20 days in the cerebral cortex (Vanier et al., 1971 and DeRaveglia et al., 1972) and during the first three weeks postnatally in the hippocampus (Irwin and Irwin, 1979). In the brain stem, the accumulation is slow and shows a broad peak from 10 days to 2 months (Merat and Dickerson, 1973). No published reports are available regarding the accumulation of gangliosides in the corpus striatum and the hypothalamus. Since Seyfried et al. (1982) have demonstrated that specific gangliosides appear to be concentrated in specific cell types in the cerebellum (their findings suggest that  $GD_{1a}$  is concentrated in Purkinje cells and  $GD_3$  appears to be enriched in reactive glial cells), studies on the alteration of synthesis of gangliosides in different brain regions could permit a more precise localization of neurochemical abnormalities that may be present in FAS offspring. In addition, these studies are of potential importance as gangliosides have been postulated to be important in synaptic ion fluxes (McIlwain, 1961), synaptic connectivity and cellular interactions (Brunngraber, 1969; Barondes, 1970)

neurotransmitter uptake and binding (Brunngraber, 1969; Salvatera and Matthews, 1980), as components of receptors for neurotransmitters (Wooley and Gommi, 1966), involvement in behavior (Irwin, 1974) and as markers of different cell types (Seyfried et al., 1982).

## CHAPTER II

### MATERIALS AND METHODS

#### Animals

Female Sprague-Dawley albino rats were purchased from Holtzman (Madison, WI.).

#### Diet

Different dietary protocols were employed, i.e., normal ad libitum laboratory chow, and defined periods of maternal ethanol consumption. Except during the defined periods of ethanol consumption, all animals were given free access to water and standard laboratory chow (Purina Laboratory Chow, Purina, St. Louis, MO.) containing casein (27%), starch (59%), vegetable oil (10%), salt mixture and vitamin fortification (4%).

Maternal ethanol consumption was carried out by employing a pair feeding paradigm using the isocaloric control and alcohol diet described by Lieber and DeCarli (1974). Daily and cumulative average daily volume, calorie and alcohol consumption were recorded. The isocaloric control and alcohol diets were identical in vitamin, mineral, lipid, protein and calorie content. The only difference between the control and alcohol diets was the isocaloric substitution of ethanol in the alcohol diets for calories in the control diets otherwise supplied by maltose-dextrins.

The Lieber-DeCarli diet is commercially available (Bio-Serv, Frenchtown, N.J.) and contains a fixed alcohol content of 6.6% (v/v) in the alcohol diet. Protein, fat and carbohydrate account for respectively 18%, 35% and 47% of the calories in this diet. The composition of the control and alcohol Lieber-DeCarli liquid diets is described in Table II. Groups of animals which consumed this diet were designated C-C for control and E-E for ethanol.

#### Revised Liquid Diet

A revised liquid diet was formulated in which the protein content was increased from 18% of the total calories of the Lieber-DeCarli diet to 21% of the total calories. In this revised diet, fat and carbohydrate accounts for 29% and 50% of the total calories. In addition, seventy-five milliliters of chocolate Sego (Pet Inc., St. Louis, MO.) was included in each liter of this diet for flavoring. Calories obtained by use of Sego were taken into account when formulating the diet. The composition of the control and alcohol revised liquid diet is described in Table III. Groups of animals that consumed the diet were designated \*E-\*E for ethanol and \*C-\*C for control.

#### Administration of Diets

Female Sprague-Dawley rats were placed on the control diet three days after they were delivered to the Animal



TABLE II

Lieber DeCarli Liquid Diet

<u>Substituent</u>	<u>Control Gram/Liter</u>	<u>Control Percent Calories</u>	<u>Ethanol Grams/Liter</u>	<u>Ethanol Percent Calories</u>
Protein:		18%		18%
Casein, vitamin free:	41.4		41.4	
L-Cystine:	.5		.5	
DL-Methionine:	.3		.3	
Carbohydrates (Non-Alcoholic):		47.5%		11.7%
Maltose- Dextrins:	114.0		24.4	
Dextrose Vitamin Mixture:	5.0		5.0	
Carbohydrates (Alcoholic):		0.0%		35.8%
Ethanol:	0.0		50.0	
Fats:		35.6%		35.6%
Corn Oil:	8.5		8.5	
Olive Oil:	28.4		28.4	
Ethyl Linoleate:	2.7		2.7	
Salt Mixture:	10.0		10.0	

TABLE III

Revised Liquid Diet

<u>Substituent</u>	<u>Control Gram/Liter</u>	<u>Control Percent Calories</u>	<u>Ethanol Grams/Liter</u>	<u>Ethanol Percent Calories</u>
Protein:		21.1%		21.1%
Casein, vitamin free:	47.95		47.95	
L-Cystine:	.63		.63	
DL-Methionine:	.37		.37	
Sego:	3.75		3.75	
Carbohydrates (Non-Alcoholic):		49.6%		13.8%
Maltose- Dextrins:	114.00		24.40	
Dextrose Vitamin Mixture:	5.00		5.00	
Sego:	15.00		15.00	
Carbohydrates (Alcoholic):		0.0%		35.8%
Ethanol:	0.00		50.00	
Fats:		29.3%		29.3%
Corn Oil:	4.37		4.37	
Olive Oil:	27.70		27.70	
Ethyl Linoleate:	2.60		2.60	
Sego:	.75		.75	
Salt mixture:	10.00		10.00	

Research Facility. After a period of one week of adjustment to the liquid diets, at least half of the female rats were maintained on the corresponding, isocaloric (6.6%, v/v) ethanol liquid diet. The control and ethanol diets were pair-fed to rats for approximately two months prior to conception and throughout gestation. At parturition, dams fed the alcohol diets were withdrawn from ethanol over a three day period, during which withdrawal was moderated by half strength (3.3%, v/v) alcohol diet and free access to standard laboratory chow. During the same three day withdrawal period, control dams were given access to laboratory chow in addition to a control liquid diet. After three days, all rats were fed standard laboratory chow exclusively.

#### Protocol for Cross-fostering of Animals

In cross-fostering studies, the pups born to mothers who were maintained on the control or alcohol liquid diets were cross-fostered on the third postnatal day. Thus, these pups were separated from their biological mothers and were subsequently reared by surrogate mothers that consumed standard laboratory chow ad libitum during the entire experiment--namely, two months prior to conception and throughout gestation. Groups of animals in this study were designated \*E-Ch for the ethanol cross-fostered and \*C-Ch for the control cross-fostered.

### Protocol for Reverse Cross-Fostering of Animals

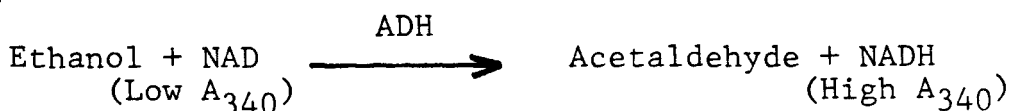
In the reverse cross-fostering studies, pups that were born to mothers that consumed laboratory chow during the entire experiment were reared from the third postnatal day by mothers who had previously been maintained on the revised control or alcohol liquid diets on a chronic basis. Animals in this particular group of study were designated Ch-\*E for ethanol and Ch-\*C for control.

Table IV summarizes the different groups of animals used in studies presented in this dissertation.

### Determination of Blood Ethanol Levels

Blood ethanol levels were determined using an enzymatic kit (Sigma, St. Louis, MO.). Control and alcohol animals were bled from the tail and the blood was collected in heparinized tubes (10 mg of heparin/ml of whole blood), stoppered immediately and placed on ice.

The procedure used in the enzymatic kit is based upon the method of Bonnichsen and Theorell (1951) and utilizes alcohol dehydrogenase (ADH) and nicotinamide adenine dinucleotide (NAD). The enzyme ADH catalyzes the conversion of ethanol to acetaldehyde in the following manner:



The formation of acetaldehyde is favored when the reaction

TABLE IV  
Offspring Groups

Diet Consumed by Biological (Gestational) Mother	Diet Consumed by the Nursing (Rearing) Mother Prior to the 3rd Postnatal Day	Abbreviation for Animal Group
Original Liquid Diet		
Ethanol (E)	Ethanol (E)	E-E
Control (C)	Control (C)	C-C
Revised Liquid Diet (*)		
Ethanol (*E)	Ethanol (*E)	*E-*E
Control (*C)	Control (*C)	*C-*C
Ethanol (*E)	Chow (Ch)	*E-Ch (Cross-fostered)
Control (*C)	Chow (Ch)	*C-Ch (Cross-fostered)
Chow		
Chow (Ch)	Ethanol (*E)	Ch-*E (Reverse cross-fostered)
Chow (Ch)	Control (*C)	Ch-*C (Reverse cross-fostered)

Note: All rat mothers consume chow exclusively after the 3rd postnatal day.

takes place at approximately pH 9.0. Furthermore, the acetaldehyde formed is trapped with semicarbazide, thus causing the reaction to proceed nearly to completion. The increase in absorbance at 340 nm, which occurs when NAD is converted to NADH is an accurate measure of the amount of ethanol present.

Determinations of ethanol levels in the blood were made on rats maintained chronically on an alcohol diet three hours after the introduction of a fresh ration of the diet.

### Isotope

L-[1-<sup>14</sup>C]-Fucose (59mCi/mmole) and L-[1-<sup>3</sup>H]-fucose (5.4 Ci/mmole) were purchased from Amersham/Searle (Arlington Heights, IL.). N-[Acetyl-1-<sup>14</sup>C]-D-mannosamine (18mCi/mmole) and N-[acetyl-<sup>3</sup>H]-D-mannosamine (3Ci/mmole) were purchased from International Chemical and Nuclear Corporation (Irvine, CA.).

Fucose was selected as a precursor of glycoproteins and N-acetyl-D-mannosamine as a precursor of the sialic acid component of gangliosides. The specific activity of L-[1-<sup>3</sup>H]-fucose and N-[acetyl-<sup>3</sup>H]-D-mannosamine were adjusted to 200 mCi/mmole and 110 mCi/mmole, respectively, with non-radioactive fucose and N-acetyl-mannosamine. Fucose was selected as a precursor of SPM glycoproteins because of its specificity for labelling glycoproteins (Quarles and Brady, 1971) and because approximately 85% of SPM glycoproteins

contain fucose (Zanetta et al., 1977). N-Acetyl-mannosamine was used as a precursor of sialic acid in the ganglioside studies because of the specificity with which it labels the sialic acid moiety of gangliosides (Quarles and Brady, 1977; Yohe and Rosenberg, 1977).

#### Administration of Isotope

An isotope solution containing either 30  $\mu$ Ci of a [ $^3\text{H}$ ] precursor or 10  $\mu$ Ci of [ $^{14}\text{C}$ ] precursor in 10  $\mu$ l of 0.85% (w/v) NaCl was prepared. Under mild ether anesthesia, the isotopically labelled solution was administered intracerebrally in young rats either by means of a single 10  $\mu$ l injection along the midline of the skull or in two divided injections each 5  $\mu$ l, on either side of the midline of the skull. Previously, this laboratory has shown that similarly injected dye solutions appeared to be concentrated intra-ventricularly shortly after injection.

#### Schedule of Isotopic Injection and Subsequent Membrane Fractionation

Pups were injected with either the radioisotopically labelled glycoprotein precursors or the radioisotopically labelled ganglioside precursors and were sacrificed eighteen hours later. Different specimen types were isolated according to a schedule appropriate to the aims of each particular experimental sequence.

Table V gives the schedule of isotopic injections

TABLE V

Schedule of Isotope Injection and Specimen Isolation

<u>Animal</u>	<u>Specimen Isolated</u>	<u>Isotope received (in 10 microliters saline)</u>	<u>Ages of injection (Days postpartum)</u>	<u>Ages of specimen isolated (18 hr. after injection) (days postpartum)</u>
rat	Synaptic plasma membrane	30 $\mu\text{Ci}$ of [ $^3\text{H}$ ]-fucose or N-acetyl-mannosamine; 10 $\mu\text{Ci}$ [ $^{14}\text{C}$ ]-fucose and N-acetyl-mannosamine	9, 16, 23 and 30	10, 17, 24 and 31
rat	Microsomal	10 $\mu\text{Ci}$ of [ $^{14}\text{C}$ ]-N-acetyl-mannosamine	9, 16 and 23	10, 17 and 24
rat	Synaptosomal	10 $\mu\text{Ci}$ of [ $^{14}\text{C}$ ]-N-acetyl-mannosamine	9, 16 and 23	10, 17 and 24
rat	Hypothalamus, Hippocampus, Brain Stem, Corpus Striatum, Cerebellum, Cerebral Cortex	30 $\mu\text{Ci}$ of [ $^3\text{H}$ ]-or 10 $\mu\text{Ci}$ of [ $^{14}\text{C}$ ] N-acetyl-mannosamine	9, 16 and 23	10, 17 and 24

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and also the type of specimen isolated after the isotopic injections. During each of the different studies performed, the litter size of pups was adjusted to 10 pups per litter, three days after birth and all the litters were weaned at approximately 21 days.

In most of the studies, six pairs of animals (6 control and 6 ethanol) were included for an individual data point. Half of the animals received a [ $^3\text{H}$ ] isotopic precursor while the other half received a [ $^{14}\text{C}$ ] isotopic precursor. In addition, the assignment of the [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ] precursors to control and ethanol animals were varied in order to rule out an isotopic effect. The only exception to this schedule were in studies of microsomal and synaptosomal membrane fraction gangliosides. In these studies both the control and ethanol animals received the same [ $^{14}\text{C}$ ] isotopic precursor.

#### Isolation of Purified Synaptic Plasma Membrane (SPM) from Whole Brain

Animals were sacrificed by decapitation. Whole brains were rapidly dissected and weighed. Brains were kept at  $0^\circ\text{C}$  and SPM isolation followed immediately. Purified whole brain synaptic plasma membranes were isolated according to the method of Cotman and Matthews (1971), employing ultra-centrifugation equipment available in this laboratory. Throughout the isolation procedure, the tissue was maintained between  $0$  and  $4^\circ\text{C}$ .

A 20% homogenate of whole brain was prepared in ice-cold 0.32 M (w/v) sucrose in a powered tissue homogenizer (Kontes Glass Co., Vinland, N.J.) equipped with a conical teflon-coated pestle. This homogenate was then diluted to a 10% homogenate (w/v) with 0.32 M (w/v) sucrose for centrifugation. One ml of this homogenate was reserved for subsequent analysis. The remaining 0.32 M sucrose homogenate was centrifuged in a Sorvall RC-5B refrigerated super speed centrifuge using a SS-34 rotor at 1000-1100 x g or 3,000 revolutions per minute (rpm) for 5 minutes to give a crude nuclear fraction (P1). The supernatant (S1) was transferred to another centrifuge tube and centrifuged at 13,700 rpm (17,000 x g) for about 10 minutes. The crude mitochondrial pellet (P2) was resuspended in 15 ml of 10% (w/v) sucrose by hand homogenization (Dounce homogenizer, Kontes Glass Co., Vinland, N.J.) and centrifuged at 11,000 rpm (11,000 x g) in a Sorvall centrifuge for twenty minutes. This washing of the crude mitochondrial pellet (P2) was repeated two more times. The P2 pellet was then suspended in 15 ml of a 10% (w/v) sucrose solution by hand homogenization and layered on a discontinuous Ficoll-sucrose gradient, which consisted of 10 ml layers of 13% (w/v) Ficoll (Pharmacia, Piscataway, N.J.) in 0.32 M sucrose and 7.5% (w/v) Ficoll in 0.32 M sucrose. The tubes were centrifuged at 22,000 rpm (63,581 x g) for 45 minutes in a SW 27 rotor, and a synaptosomal fraction was obtained at the interface of the 7.5%-13% Ficoll-sucrose layers. The

synaptosome band or interfacial material was removed by means of a Pasteur pipette, diluted with 4 volumes of 10% sucrose and pelleted at 24,250 rpm for 30 minutes. The synaptosomal pellet was then resuspended in a small volume of 10% sucrose and osmotically shocked in 5 volumes of 6 mM Tris, pH 8.1, for one and a half hours in the cold. After osmotic shock the fraction was concentrated by centrifugation for 15 minutes at 20,300 rpm (54,500 x g) in a SW 27 rotor. The pellet was resuspended in 10% sucrose and layered over a discontinuous sucrose gradient. The discontinuous sucrose gradient consisted of successive 7.5 ml layers of 25.0, 32.5, 35.0 and 38% sucrose (w/v). Centrifugation was carried out for 1.5 hours at 22,000 rpm in a SW 27 rotor. The synaptic plasma membrane fraction was recovered at the 25.0-32.5% sucrose interface by a Pasteur pipette and was diluted with 0.1 mM EDTA and centrifuged at 24,250 rpm (106,000 g) for 30 minutes. The pellet was then resuspended in a final volume of one to two ml ice-cold 10% sucrose solution and kept frozen at or below -20°C until subsequent assays and analytical procedures were performed.

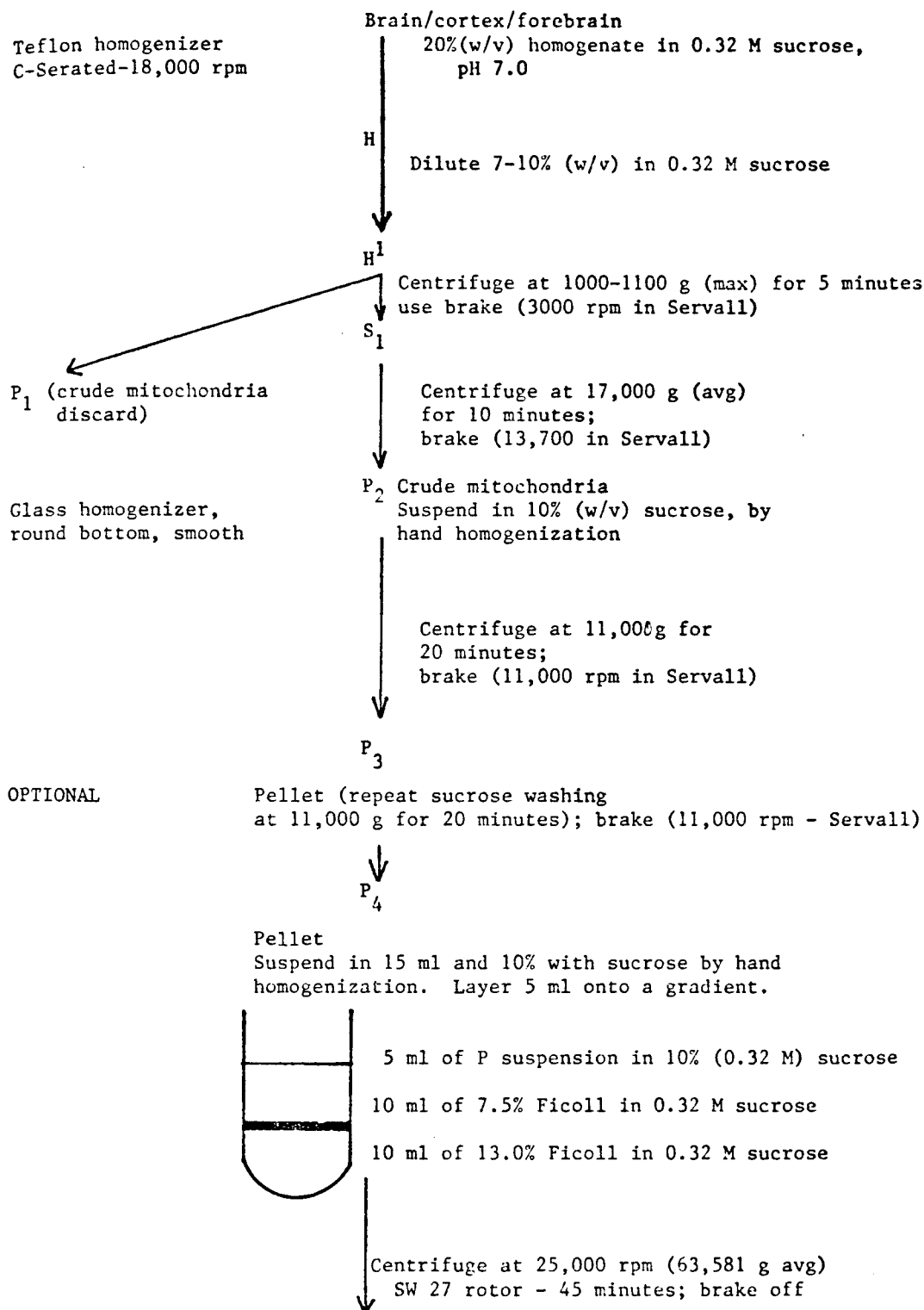
In the discontinuous sucrose density gradient, any myelin contamination was recovered in the 10%-25% interface while the 38% sucrose layer effectively resolved purified mitochondria from most membranes.

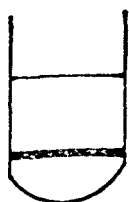
Figure 1 is a schematic representation of the procedure for the isolation of synaptic plasma membranes from

Figure 1

## Isolation of Synaptosomal Plasma Membranes

Cotman &amp; Matthews BBA 249 (1971) 380-394





0.32 M sucrose  
7.5% Ficoll - 0.32 M sucrose  
13.0% Ficoll in 0.32 M sucrose

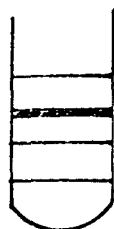
Synaptosomal material is found at  
7.5/13.0% Ficoll-sucrose interface.

Remove interfacial material.  
Dilute with 4 volumes of 10% sucrose (0.32M)  
Centrifuge at 106,000 g max for 30 minutes

Suspend the pellet in a small volume of 10% sucrose. Osmotically shock pellet in 5 volumes of 6 mM Tris, pH 8.1 for 1.5 hours (in the cold)

Centrifuge for 15 minutes at 54,500 g average.

Resuspend the pellet in 10% sucrose and layer it over:



10% sucrose-pellet suspension  
25.0% sucrose  
32.5% sucrose      5 ml each  
35.0% sucrose  
38.0% sucrose

Centrifuge for 1.5 hours at 25,000 rpm in the SW 27 rotor  
Remove the bands and dilute each with 0.1mM EDTA  
Pellet each band at 106,000 g max for 30 minutes  
Suspend each pellet in 10% sucrose for assays.

whole brain.

#### Isolation of Microsomal Membrane Fraction from Whole Brain

In the isolation procedure described above the supernatant S2 was spun down at 24,250 rpm (106,000 x g) for one hour in a SW 27 rotor to give the P2B or microsomal pellet. The pellet was then dispersed in 10% sucrose and spun down at 24,250 rpm (106,000 x g) for half an hour. This step was repeated an additional time and then the microsomal pellet was suspended in one or two ml of 10% sucrose and frozen.

#### Isolation of Synaptosomal Membrane Fraction from Whole Brain

The synaptosomal membrane fraction was isolated by the method of Cotman and Matthews (1971) as described previously. The synaptosomal band obtained at the 7.5%-13% Ficoll sucrose interface was collected, suspended in 10% sucrose and pelleted at 24,250 rpm (106,000 x g) in a SW 27 rotor for 30 minutes. This procedure was repeated and the resulting pellet was resuspended in 1 ml of 10% sucrose and frozen.

#### Isolation of Different Brain Regions for Ganglioside Analysis

Animals were sacrificed by decapitation. Whole brains were carefully removed, blotted and chilled. Dissections were performed on an ice-cooled glass plate according to the

method of Glowinski and Iversen (1966). Six brain regions--hypothalamus, hippocampus, cerebellum, brain stem, corpus striatum and the cerebral cortex were dissected on dry-ice. The corpus striatum included the putamen nucleus, caudate nucleus and the globus pallidus nucleus. The cortex included the white and grey matter of the cerebral cortex.

Dissected tissues were weighed immediately and homogenized in ice-cold 0.32 M sucrose with a polytron tissue homogenizer (Brinkman Instruments, Westbury, N.Y.). An aliquot of the homogenate was reserved for scintillation counting and the rest of the sample was lyophilized.

#### Assays and Analytical Procedures

Samples of each brain homogenate and suspensions of microsomal, synaptosomal and synaptic plasma membrane fractions were used for liquid scintillation counting and protein assays. The remaining aqueous suspensions of the different membrane fractions were lyophilized. Lyophilized material was then committed for delipidation of protein, in preparation for subsequent SDS polyacrylamide gel electrophoretic characterization of SPM proteins. Similarly, lyophilized material from other membrane fractions and brain regions which were labelled with N-acetylmannosamine was committed for extraction of lipids and subsequent ganglioside extraction and analysis. The specific assays and analytical procedures are described in detail below.

### Liquid Scintillation Counting Techniques

The radioactivity of [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ] in a number of different sample preparations was measured by liquid scintillation counting techniques. Regardless of the techniques employed to extract and solubilize the radioactivity present in such samples, radioactivity was counted using a toluene based liquid scintillation fluor system. The only exception was with TLC scrapings of ganglioside samples. In this case, the scintillation fluor system used was Aquasol.

### Preparation of Toluene Based Liquid Scintillation Fluor System

To one gallon scintillation grade Toluene (Scintillar, Malinckrodt, Inc., St. Louis, MO.) was added 200 ml Toluene, 16 grams PPO, (2,5-Diphenyloxazole), scintillation grade (Amersham/Searle, Arlington Heights, IL.) and 0.4 gram POPOP, (p-Bis[2-(5-Phenyloxazolyl)]-benzene), scintillation grade (New England Nuclear, Boston, MA.).

Samples were solubilized with NCS Tissue Solubilizer (Amersham/Searle, Arlington Heights, IL.) prior to the addition of fluor, except for electrophoresed sodium dodecyl sulfate polyacrylamide gel protein bands and TLC plate scrapings of individual gangliosides. Radioactivity in separated SDS polyacrylamide gel protein bands was eluted by digestion of gel slices in Protosol Tissue Solubilizer (New England Nuclear, Boston, MA.) (Protosol:water, 9:1, v/v) at room temperature. The radioactivity in individual gangliosides



was determined by adding one ml of distilled water to the tlc scrapings containing the gangliosides, followed by sonication. Ten ml of liquid scintillation Aquasol (New England Nuclear, Boston, MA.) was then added to the sonicated samples.

To brain homogenate and different membrane fraction samples suspended in a maximum aqueous volume of 100  $\mu$ l in a liquid scintillation counting vial were added one ml of NCS, 100  $\mu$ l of 0.1 N acetic acid, and 10 ml of fluor, in this order.

All samples were counted in a Beckman LS7500 (micro-processor controlled) liquid scintillation counter (Beckman Instruments, Ins., Fullerton, CA.) in accordance with a counting protocol previously developed and in use in this laboratory. The predetermined counting protocol permitted counting of [ $^{14}\text{C}$ ] and dual labelled [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ] samples. A quench correction curve, previously determined in this laboratory was used to determine counting efficiencies. At the settings used, less than 1.0% of actual [ $^3\text{H}$ ] activity spilled into the [ $^{14}\text{C}$ ] counting channel while approximately 10.0% of the actual [ $^{14}\text{C}$ ] activity spilled into the [ $^3\text{H}$ ] counting channel. Observed cpm (counts per minute) data were automatically converted to dpm (disintegrations per minute) by means of a computer attached to the scintillation counter.

#### Lowry Protein Assay

Protein content of aliquots of brain homogenate, synaptic plasma membrane, microsomal and synaptosomal fractions was determined by a modification of the protein assay of Lowry, Rosenbrough, Farr, and Randall (1951).

### Preparation of Solutions

#### 1. 1 N Sodium Hydroxide

40 grams of NaOH (Mallinckrodt, St. Louis, MO.) was dissolved in distilled water to a volume of one liter.

#### 2. 2% (w/v) Sodium Potassium Tartarate

10 grams of  $\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$  crystals (Mallinckrodt, St. Louis, MO.) were dissolved in distilled water to a volume of 500 ml.

#### 3. 1% (w/v) Cupric Sulphate

10 grams of  $\text{CuSO}_4$  (Mallinckrodt, St. Louis, MO.) were dissolved in distilled water to a volume of 1 liter.

#### 4. 2% (w/v) Sodium Carbonate

10 grams of  $\text{Na}_2\text{CO}_3$  (Anhydrous, Mallinckrodt, St. Louis, MO.) were dissolved in distilled water to a volume of 500 ml.

#### 5. 1 N Phenol Reagent

100 ml of a commercially available 2N preparation of Folin and Ciocalteu Phenol Reagent (Harleco, Philadelphia, PA.) was dissolved in distilled water to a volume of 200 ml and refrigerated.

#### 6. 0.1% (w/v) Human Serum Albumin

10 mg of Human Serum Albumin (HSA) (Sigma Chemical Co., St. Louis, MO., stored dessicated, 0-4°C) were dissolved in distilled water to a volume of 10 ml and refrigerated.

Aqueous samples of material to be assayed in less than 60  $\mu$ l were added to small test tubes. To each sample was added 100  $\mu$ l of 1 N NaOH. Each test tube was vortexed. Following a 30 minute incubation at room temperature, 1 ml of a mixture of NaKTartarate-CuSO<sub>4</sub>-Na<sub>2</sub>CO<sub>3</sub> (0.1:0.1:10, v/v/v) prepared by addition of reagents in this order was added to each sample. Test tubes were vortexed and incubated at room temperature for 10 minutes. To each test tube was added 100  $\mu$ l of 1 N Phenol Reagent, followed by immediate vortexing of each test tube. The test tubes were allowed to stand for 30 minutes at room temperature. In the presence of protein, a blue color developed. The absorbance was measured spectrophotometrically against a reagent blank at 700 nm. A standard curve was prepared for each assay using 5 to 60  $\mu$ l of a 1  $\mu$ g/ $\mu$ l HSA standard solution.

### Separation of SPM Protein and Glycoprotein

#### SDS Polyacrylamide Gel Electrophoresis

Separation of SPM proteins and glycoproteins by SDS polyacrylamide gel electrophoresis involved a number of steps, i.e., delipidation of SPM protein, solubilization of protein, gel preparation, actual electrophoresis, fixation, staining, and destaining of gels, densitometric

analysis of gel scans, and the recovery of radioactivity in separated protein.

Lyophilized SPM material was delipidated with diethyl ether:ethanol (3:2, v/v) according to the procedure of Greenfield, Norton and Morell (1971). One ml of diethyl ether:ethanol (3:2, v/v) was added to a sample of lyophilized SPM fraction in a test tube and vortexed. After centrifugation for 10 minutes at 2000 to 2500 rpm in a table top centrifuge the ether:ethanol supernatant was drawn out by a Pasteur pipette and discarded. Delipidation was similarly repeated twice. Samples were dried carefully under a stream of nitrogen and immediately solubilized.

Delipidated lyophilized synaptic plasma membrane protein was solubilized according to the procedure of Quarles et al. (1973b). Protein was solubilized and vortexed in solubilizing solution for a minimum of 1 to 2 hours. Solubilizing solution was prepared fresh. Solubilizing solution contained 2.5% (w/v) sodium dodecyl sulphate (SDS), 1.0% (w/v) sodium carbonate, and 10.0% (v/v)  $\beta$ -mercaptoethanol.

#### Preparation of Solubilizing Solution

To 100 ml of a stock solution of 5.0% (w/v) SDS (technical grade, Matheson, Coleman and Bell, Ohio) (50 grams SDS per liter) was added 20 ml of a stock solution of 10.0% (w/v)  $\text{Na}_2\text{CO}_3$  (Mallinckrodt, St. Louis, MO.) (50 grams  $\text{Na}_2\text{CO}_3$  per 500 ml) and 20 ml of  $\beta$ -mercaptoethanol (Matheson,

Coleman and Bell, Ohio) with dilution by distilled water to a volume of 200 ml.

Upon completion of solubilization, solubilized protein was dialyzed overnight against a fresh dialyzing solution. The dialyzing solution contained 0.1% (w/v) SDS, 0.01 M sodium phosphate buffer (pH 7.2), 1.6 M urea, and 0.05% (w/v) dithiothreitol. Sufficient dialyzing solution was prepared to provide 100 to 200 ml dialyzing solution per ml of solubilized protein solution.

### Preparation of Solutions

#### 1. Phosphate Buffer

1 M Phosphate Buffer, pH 7.2

39.0 grams Sodium Monobasic Phosphate·H<sub>2</sub>O (NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O) (Mallinckrodt, St. Louis, MO.) and 192.0 grams Sodium Dibasic Phosphate·7H<sub>2</sub>O (Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O) (Mallinckrodt, St. Louis, MO.) were dissolved in distilled water to a volume of 1 liter.

#### 2. Dialyzing Solution

To 20 ml of stock solution of 5.0% (w/v) SDS was added 10 ml of 1 M Sodium Phosphate Buffer, 200 ml of a stock solution of 8 M Urea (480.5 grams Urea per liter), and 500 mg Dithiothreitol with dilution by distilled water to a volume of 1 liter.

The optimal amount of protein to be electrophoresed

on a given gel for visualization of the stained protein and resolution of bands was 150-200  $\mu$ g. Larger amounts were electrophoresed to assess the distribution of radioactivity on electrophoresed gels. SDS polyacrylamide gels were prepared and SPM protein electrophoresed according to the procedure of Druse et al. (1974).

### Preparation of Solutions

#### 1. Gel Solution A1

To 8 ml of a stock solution of 5.0% (w/v) SDS was added 40 ml of 1 M Sodium Phosphate Buffer (pH 7.2) with dilution by distilled water to a volume of 180 ml. Solution A1 was made fresh monthly.

#### 2. Gel Solution A2

40 grams Acrylamide (Eastman Kodak Co., Rochester, N.Y.) and 1.040 grams N,N'-methylenebisacrylamide (Eastman Kodak Co., Rochester, N.Y.) were dissolved in distilled water to a volume of 180 ml. Solution A2 was made fresh monthly.

#### 3. Gel Solution B

90 mg Ammonium Persulfate and 50 ml of N,N,N',N'-Tetramethylethylenediamine (TEMED), (Eastman Kodak Co., Rochester, N.Y.) were dissolved in distilled water to a volume of 10 ml. This solution was made fresh immediately before making gels.

#### 4. Electrophoresis Chamber Buffer Solution

Buffer Solution 0.1% (w/v) SDS and 0.1 M Phosphate Buffer (pH 7.2) was made by adding 20 ml of a stock solution of 5.0% (w/v) SDS to 100 ml of 1.0 M Phosphate Buffer (pH 7.2) with dilution by distilled water to a volume of 1 liter. This solution was made fresh in sufficient volume for adequate electrophoresis buffering capacity.

Gels were prepared by quickly mixing 22.5 ml each of gel solutions A1 and A2 with 5 ml of gel solution B. The final mixture was deaerated for 30 to 60 seconds. Approximately 2.5 ml of this mixture was quickly and carefully pipetted into previously cleaned glass gel tubes (internal diameter 6 mm). Gels were immediately overlayersed with a few drops of water. Optimal polymerization was indicated by appearance, disappearance and reappearance of a sharp interfacial difference in refractive index between the gel solution and the overlayersed water, visible within 30 minutes. Polymerized gels of approximately 5 to 6 cm in length were used within a day of preparation. Dialyzed, solubilized protein was electrophoresed in electrophoresis chambers with sufficient buffer present. Gels were electrophoresed usually for approximately 24 hours. Maximum voltage never exceeded 50 volts and maximum amperage per gel never exceeded 6 to 7 ma. Bromophenol Blue was used as a tracking dye migrating ahead of any protein. Gel

electrophoresis was terminated when the dye front was within 5 mm of the gel tube end. Gel material below the lower half of the dye was discarded after completion of electrophoresis.

Gels were rapidly removed from gel tubes and were fixed, stained and destained according to the procedure of Greenfield et al. (1971).

### Preparation of Solution

#### 1. Fixing Solution

Methanol:Glacial Acetic Acid:Distilled Water  
(45:10:45, v/v/v)

#### 2. 1.0% (w/v) Fast Green Staining Solution

10 grams Fast Green Dye dissolved in Fixing Solution,  
Methanol:Acetic Acid:Water (45:10:45, v/v/v) to a  
volume of 1 liter.

Staining solution was mixed and filtered prior to use.

#### 3. Destaining Solution

Identical to Fixing Solution

Electrophoresed gels were fixed in capped test tubes and gently shaken for variable periods ranging from 3 to 4 days minimum with frequent changes of fixing solution. Fixation was continued until optimum clarity of gels were observed and the appearance of white flocculent material was minimal. Gels were stained with 1% (w/v) Fast Green for 1.5 to 2.0 hours at 37°C in a water bath. At the end



of the staining period gels were immediately rinsed with tap water, distilled water and fixing solution. They were kept in capped test tubes and gently shaken in a shaker with frequent changes of fixing solution.

The radioactivity in separated SPM protein bands from electrophoresed SDS polyacrylamide gels was eluted by digestion of gel slices in Protosol:water (9:1, v/v). Digestion at room temperature was continued for approximately three days with periodic vortexing of minced gel slices before fluor was added to scintillation vials.

#### Extraction of Gangliosides From Microsomal, Synaptosomal and Synaptic Plasma Membrane Fractions

Gangliosides were extracted from total lipids extracts of microsomal, synaptosomal and synaptic plasma membrane fractions according to the procedure of Suzuki (1964) with modifications.

#### Reagents

1. Chloroform ( $\text{CHCl}_3$ )-Methanol (MeOH) 2:1 (v/v).
2. Chloroform-Methanol 1:2, with 5% water.
3. 0.1 N KCl.

0.74 grams of KCl (Mallinckrodt, St. Louis, MO.) was dissolved in 100 ml of distilled water.

4. Theoretical Upper Phase (TUP) with salt.

Chloroform, methanol and water containing 0.74% KCl was mixed in the following proportions:  $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}$ , 3:48:47, with water containing 0.74% KCl.

5. Theoretical Upper Phase without salt.

Chloroform, methanol and water was mixed in the following proportions:  $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}$ , 3:48:47.

6. Resorcinol Stock Solution (Mallinckrodt, St. Louis, MO.).

2 grams resorcinol was dissolved in 100 ml of distilled water and stored at 4°C in a dark bottle.

7. Resorcinol-HCl reagent.

10 ml of stock resorcinol solution, 80 ml of concentrated hydrochloric acid (HCl, Mallinckrodt, St. Louis, MO.) and 0.25 ml of 0.1 M copper sulfate ( $\text{CuSO}_4$ ) was brought to a volume of 100 ml with distilled water. The solution was prepared at least four hours prior to use and was stored in a dark bottle no longer than two weeks at 4°C.

8. Butanol-butyl acetate. (15:85).

15 ml of butanol (Mallinckrodt, St. Louis, MO.) and 85 ml butyl acetate (Mallinckrodt, St. Louis, MO.) was mixed and stored at room temperature.

Total lipids from lyophilized synaptic plasma membranes, microsomal and synaptosomal membranes were extracted by the method of Folch-Pi et al. (1957). Twenty volumes of chloroform-methanol (2:1) was added to each lyophilized membrane sample. Samples were alternately vortexed and

allowed to sit several times over a period of about 30 minutes, and then centrifuged at 2500 rpm in a table top centrifuge for approximately 15 minutes. The supernatant, containing the membrane lipids was drawn off with a Pasteur pipette and collected. This process was repeated once with chloroform-methanol (2:1) and then with chloroform-methanol (1:2) with 5% water. The extracts from each sample were combined and dried by means of a rotary evaporator (Buchi, Switzerland). The dried filtrate of total lipid extract was resolubilized in 30 ml of chloroform-methanol (2:1) and transferred to 50 ml conical stoppered partitioning tubes for the subsequent partitioning steps with repeated washings.

To the 30 ml of lipid extract in the partitioning tube was added 6 ml of 0.1N KCl and the tubes were stoppered and vigorously shaken by hand for about 20 to 30 minutes. The tubes were then stoppered and centrifuged at 1500 to 2000 rpm in a cold centrifuge (International Centrifuge, IEC, Needham, MA.) for a minimum of 10 minutes. After centrifugation, the upper phase was carefully drawn off with a Pasteur pipette and collected in a round bottom flask. This process was repeated, once with theoretical upper phase in KCl, and then with theoretical upper phase without salt. The pooled upper phases from each sample were then taken to dryness in a rotary evaporator for the next alkaline hydrolysis step.

Alkaline hydrolysis was carried out on the dried

upper phases in a round bottom flask. Three ml of chloroform was added to the flask, making sure that the contents on the sides of the flask was rinsed down. Three ml of 0.6N NaOH in methanol was then added and the contents of the flask was gently swirled every 15 minutes during the next 60 minutes. At the end of the hour, the reaction was stopped by adding 0.15 ml of concentrated hydrochloric acid. The contents of the flask was carefully taken down to dryness for the subsequent desalting step.

#### Desalting of Upper Phase

The dried upper phases after alkaline hydrolysis were desalted by the method of Williams and McCluer (1980) using Sep-Pak cartridges (Waters Associates, Milford, MA.). The Sep-Pak cartridge was fitted with a three-way stopcock (Pharmaseal Inc., Toa Alto, Puerto Rico). Samples and solvents were applied to the cartridge by means of a 30 ml syringe (Pharmaseal Inc., Toa Alto, Puerto Rico). Before applying the sample to the Sep-Pak cartridge, the cartridge was washed alternately with 10 ml of methanol and 20 ml of chloroform-methanol (2:1), three times. A final wash of 10 ml of methanol was done and this was followed by an equilibration wash of the cartridge with 10 ml of theoretical upper phase containing 0.1 M KCl.

The combined upper phases after the partitioning and alkaline hydrolysis step was brought to a final con-

centration of 0.1 M KCl and applied to the Sep-Pak cartridge with slight pressure. The eluate was collected in a glass tube and reapplied to the cartridge twice. Ten ml of water was added, to wash the salts from the cartridge. Gangliosides were eluted from the cartridge with the addition of 15 ml of methanol and collected in a round bottom flask. The contents were dried by means of a rotary evaporator under vacuum and the gangliosides were taken up in a known volume (between 100-500  $\mu$ l) of chloroform-methanol (2:1). The ganglioside extract was kept at 4°C until further separation of individual gangliosides by thin layer chromatography.

#### Extraction of Gangliosides from Different Brain Regions

Gangliosides were extracted from different brain regions by the method of Irwin and Irwin (1979) with modifications. Lipids were extracted by adding 2 ml of chloroform-methanol, 2:1 (v/v) to each lyophilized brain sample. Samples were alternately vortexed and allowed to sit several times over a period of about 30 minutes. Samples were centrifuged at 2500 rpm in a table-top centrifuge for approximately 15 minutes. The supernatant containing the lipid was drawn off with a Pasteur pipette and collected separately. The pellet was extracted once with 2 ml of chloroform-methanol 1:2 (v/v) and then again with 2 ml of chloroform-methanol 2:1 (v/v) in the manner described above. The lipid extract from each step was combined and dried

under nitrogen. The dried total lipid extract of each sample was reconstituted in 1 ml of chloroform-methanol 2:1 (v/v).

The reconstituted total lipid extract was layered on a 2 cm-long bed of silicic acid (Unisil, 100-200 mesh, Clarkson Chemical Co., Williamsport, PA.) on a base of glass wool in a Pasteur pipette (5mm i.d.) for the separation of non-ganglioside lipids from the gangliosides. Non-ganglioside (neutral) lipids were eluted from the Unisil column by adding 1 ml of chloroform-methanol 2:1 (v/v) twice. The eluate was saved for further extraction. Gangliosides were eluted from the Unisil column by adding 2 x 1 ml of chloroform-methanol-water 50:50:15 (v/v/v). With the second rinse, the silicic acid bed was stirred vigorously with a glass rod and gentle pressure was applied to the column.

The non-ganglioside lipid fraction in chloroform-methanol, 2:1 (v/v) was dried under nitrogen and dissolved in 0.5 ml of chloroform. This fraction was layered on a second Unisil column prepared in the manner described previously. The column was washed by adding 1 ml of chloroform three times to elute cholesterol. Neutral glycolipids and phospholipids were separated from the column by the method of Vance and Sweeley (1967). First, the neutral glycolipids were eluted from the column by adding 6 ml of acetone-methanol, 9:1 (v/v). The eluate was

collected and discarded. Next, the remaining gangliosides were eluted from the column by adding 2 ml of chloroform-methanol-water, 50:50:15 (v/v/v), and was collected. This ganglioside eluate was combined with the ganglioside fraction obtained from the first Unisil column, and dried under a stream of nitrogen. The gangliosides were reconstituted in a known volume of chloroform-methanol, 2:1 (v/v) for subsequent TLC separation and analysis.

Figure 2 is a schematic representation of the procedure for the extraction of gangliosides from brain regions.

#### Separation of Individual Gangliosides by Thin-Layer Chromatography

Individual gangliosides were separated from a total ganglioside extract by thin-layer chromatography. Thin-layer chromatography was done on High-Performance TLC (HP-TLC) precoated silica Gel 60 plates (E. Merck, Darmstadt, West Germany). Known quantities of ganglioside extract were spotted on the plate by means of a hamilton syringe on 8-mm lanes. Ganglioside standards GM<sub>1</sub>, GM<sub>2</sub>, GD<sub>1a</sub>, GT<sub>1b</sub> (Supelco, Bellefonte, PA.) and GM<sub>3</sub>, GM<sub>4</sub>, GD<sub>1b</sub> (gift from Dr. A. Rosenberg's laboratory) were spotted between each sample of ganglioside extract to help in identification of individual ganglioside bands. The plates were developed in a saturated tank using the solvent system chloroform-methanol-water containing 0.02% (w/v) calcium chloride, 50:45:10 (v/v/v) as described

Figure 2

Extraction of Gangliosides from Brain Regions

Extract lyophilized brain region with 2x1 ml  $\text{CHCl}_3$ :MeOH (2:1)

Extract with 2 ml  $\text{CHCl}_3$ :MeOH (1:2)

Extract with 2x1 ml  $\text{CHCl}_3$ :MeOH (2:1)

Combine extracts, dry under  $\text{N}_2$  and dissolve in 1 ml  $\text{CHCl}_3$ :MeOH (2:1)

Layer on Unisil column

Elute column with 1 ml  $\text{CHCl}_3$ :MeOH (2:1) two times. This will contain neutral lipids. Collect this extract.

6b.

Elute gangliosides from Unisil column with  $\text{CHCl}_3$ :MeOH: $\text{H}_2\text{O}$  (50:50:15) two times. Second time, stir the bed. Save this extract while continuing with 6a.

Dry down under  $\text{N}_2$

Reconstitute in 0.5 ml  $\text{CHCl}_3$

Layer on a second Unisil Column

Wash with  $\text{CHCl}_3$  (1.0 ml) three times

Elute neutral glycolipids with 6 ml Acetone:MeOH (9:1)

Elute gangliosides with  $\text{CHCl}_3$ :MeOH: $\text{H}_2\text{O}$  (50:50:15)

Combine ganglioside extracts from 6a and 6b, which are in  $\text{CHCl}_3$ :MeOH: $\text{H}_2\text{O}$  (50:50:15)

Dry under  $\text{N}_2$

Reconstitute in  $\text{CHCl}_3$ :MeOH (2:1) for spotting on TLC plates and/or Resorcinol assay.



by Yohe et al. (1980), or methyl acetate-n-propanol-chloroform-methanol-0.25% (w/v) aqueous KCl, 25:20:20:20:17 (v/v/v/v/v) as described by Zanetta et al. (1980). Individual ganglioside bands were visualized by spraying the plates with resorcinol-HCl reagent and heating at 120°C for 20 minutes under a covering glass plate. The individual ganglioside bands (resorcinol-positive spots) were densitometrically scanned at 580 nm in a Kratos Spectrodensitometer (Schoeffel Instruments, Westwood, N.J.) and the percent distribution of individual gangliosides in each sample was made using a 3390A integrator (Hewlett Packard, Palo Alto, CA.). For the determination of radioactivity in the individual gangliosides, the ganglioside extract was spotted on the plate as a long band (approximately 6 cm), together with the different standard gangliosides. After development of the plates in the solvent systems described above, one-fourth of the plate was separated and sprayed with resorcinol-HCl reagent and heated at 120°C for 20 minutes under a covering glass plate. The remaining portion of the plate was exposed to iodine vapors in a glass tank for a few minutes. Bands corresponding to each resorcinol spot were marked lightly. The iodine vapor was allowed to sublime and the individual gangliosides were then scraped from the plate. To ease scraping and to prevent loss along with airborne radioactive silica, the chromatogram was dampened lightly with water using an atomizer. The silica gel, containing sample, was placed into

scintillation vials. One ml of water was added to each vial and the contents of the vial were sonicated to solubilize the gangliosides. As stated previously, 10 ml of Aquasol was added to the vial, and the contents were vortexed, dark-adapted and counted in a liquid scintillation counter.

#### Determination of Sialic Acid

Total N-acetylneuraminic acid (NANA) in gangliosides was determined by the resorcinol-HCl reagent method of Svennerholm (1957), and the chromogen was extracted into n-butyl acetate-n-butanol as described by Miettinen and Takki-Luukkainen (1959).

Aliquots of samples containing ganglioside extracts were dried under a stream of nitrogen. One ml of water was added to each sample and the tubes were sonicated. One ml of resorcinol-HCl reagent was added to each tube and the tubes were vortexed vigorously. The tubes were heated in a boiling water bath for 15 minutes and then cooled in ice water. One ml of butyl-acetate/butanol 85:15 (v/v), was added to each tube in order to extract the color. The tubes were vortexed vigorously and centrifuged in a table-top centrifuge at 2500 rpm for about 15 minutes. In the presence of sialic acid a blue-violet color developed in the upper phase. The absorbance of this bluish upper phase was measured spectrophotometrically against a reagent blank

at 580 nm. A standard curve was prepared for each assay using 5 to 50  $\mu$ l of a 1  $\mu$ g/ $\mu$ l NANA standard solution.

### Statistical Evaluation

Values reported in tables in this dissertation are the mean values  $\pm$  the standard deviations of six animals, unless otherwise specified. P values of  $<0.05$  signify that the differences were significant. Statistical evaluations were performed using the student's 't' test.

## CHAPTER III

### RESULTS

This study examined offspring of rat mothers that were pair-fed a control (C) or 6.6% v/v ethanol (E) liquid diet in which protein accounted for either 18% (original) (C and E) or 21% revised (\*C and \*E) of the calories. In addition, groups of pups that were either cross-fostered (\*C-Ch and \*E-Ch) with chow-fed surrogate mothers or reverse cross-fostered (Ch-\*C and Ch-\*E) (offspring of chow-fed mothers fostered with \*C and \*E mothers) were examined (Refer Table IV).

Glycoprotein and ganglioside synthesis were examined in SPM and other subcellular membrane fractions in developing 10 to 31 day-old control and ethanol offspring. Ganglioside synthesis was also examined in selected brain regions.

#### Maternal Weight Gain and Diet Consumption

Both the alcohol-fed and pair-fed control rats grew from 180-200 grams to about 250 to 275 grams during the first four weeks on the revised liquid diet. During the next four week period the weights reached 330 to 380 grams. During pregnancy, the weight gain in both ethanol and pair-fed control mothers on both the original and revised liquid diets were comparable (<10%). The daily caloric,

ethanol and protein consumption by pregnant rats on the revised liquid diet was 350 kcal/kg, 17 g/kg and 18.5 g/kg, respectively, as compared to 250 kcal/kg, 10 g/kg and 13 g/kg with the original liquid diet. Blood ethanol levels were determined in randomly selected tolerant rats three hours after the introduction of a fresh ration of the revised diet and was found to be between 50-70 mg %.

### Neonatal Rats

We observed no decreased incidence of conception in alcohol-fed rats. In addition, the litter size at birth in both alcohol and pair-fed control groups were comparable. Infant or neonatal mortality was found to be negligible in both pair-fed control as well as in ethanol-exposed pups. These observations were based on approximately 45-50 litters.

### Brain Weight and Protein Content

Throughout the 10 to 24 day age range, the brain weights of ethanol and pair-fed control pups were comparable (Table VI). The only exception was noted in the reverse cross-fostered group (Ch-\*C, Ch-\*E) wherein the ethanol pups had a statistically significant ( $p < 0.05$ ) increase in the brain weight at 10 and 17 days of age.

The brain protein content doubled between 10 and 24 days of age in both control and ethanol offspring (Table VII)

TABLE VI  
Effects of Chronic Maternal Ethanol Consumption  
on Brain Weight (Grams) in Offspring

Age (Days)	Non Cross-Fostered				Cross-Fostered		Reverse Cross-Fostered	
	Original Diet		Revised Diet		Control	Ethanol	Control	Ethanol
	Control	Ethanol	Control	Ethanol				
	C-C	E-E	*C-*C	*E*-E				
		n=6		n=6		n=3		n=3
10	0.92±0.05	0.96±0.14	N.A.	N.A.	0.78±0.02	0.84±0.04	0.74±0.02	0.81±0.03
17	1.27±0.09	1.33±0.06	1.35±0.06	1.35±0.08	1.31±0.07	1.30±0.01	1.24±0.04	1.36±0.05*
24	1.38±0.10	1.54±0.10	1.47±0.08	1.48±0.08	1.48±0.03	1.46±0.03	1.39±0.03	1.49±0.08*

Each value represents the mean  $\pm$  the standard deviation. N.A. indicates that data from these animals is not available. The abbreviations C & E, \*C & \*E, and Ch stand for the diets (original control and ethanol, revised control and ethanol, and chow) that the gestational mothers-nursing mothers consumed on a chronic basis prior to and during gestation. All mothers were allowed to eat chow ad libitum during lactation. E & C pups within each group and E or C pups in different groups were compared using the student's test.

TABLE VII

**Effects of Chronic Maternal Ethanol Consumption  
on Brain Proteins (Mg/Brain) in Offspring**

Age (Days)	Non Cross-Fostered				Cross-Fostered		Reverse Cross-Fostered	
	Original Diet		Revised Diet		Control	Ethanol	Control	Ethanol
	Control	Ethanol	Control	Ethanol				
	C-C	E-E	*C-*C	*E-*E	*C-Ch	*E-Ch	Ch-*C	Ch-*E
	n=6		n=6		n=3		n=3	
10	76±8	91±19	N.A.	N.A.	104±2	100±6	85±12	98±16
17	154±8	186±17#	209±36	219±17	190±17	202±31	175±11	196±15
24	201±25	225±32	255±31	247±11	191±29	224±18	218±14	251±22

Each value represents the mean  $\pm$  the standard deviation. A # indicates that values from control and ethanol pups from the same group are different at  $p < 0.05$ . N.A. indicates that data from these animals is not available. The abbreviations C & E, \*C & \*E and Ch stand for the diets (original control and ethanol liquid, revised control and ethanol liquid, and chow) that the gestational mothers-nursing mothers consumed on a chronic basis prior to and during gestation. All mothers were fed chow ad libitum after the 3rd postnatal day.

With a few exceptions, the brain protein content was comparable in age-matched control and ethanol pups from all dietary groups.

### SPM Protein Content

The yield of SPM protein (Table VIII) also increased at least two-fold between 10 and 24 days of age. With one exception (cross-fostered groups at 17 days) there were comparable yields of SPM from ethanol and pair-fed control pups within the same experimental group. The SPM protein content of groups of offspring of rats that were fed the revised diets were comparable. In addition, the yield of SPM from these offspring are comparable to those of the offspring of chow-fed rats (Smith and Druse, in press). However, the yield of SPM from the offspring whose mothers consumed the original liquid diet (C-C, E-E) was statistically less ( $p < 0.05$ ) than the yield of SPM from the three groups of offspring that were fed the revised liquid diet.

### SPM Protein and Glycoproteins

Several double-labelled ( $^3\text{H}$  and  $^{14}\text{C}$ ) pairs of lyophilized SPMs from age-matched control and ethanol pups were combined prior to delipidation and solubilization and were separated on a 10% (w/v) polyacrylamide gel containing sodium dodecyl sulfate. The gels were sliced according to the major bands and the relative distribution of [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ]-fucose-derived



TABLE VIII

Effects of Chronic Maternal Ethanol Consumption  
on SPM Protein (Microgram/Brain) in Offspring

Age (Days)	Non Cross-Fostered				Cross-Fostered		Reverse Cross-Fostered	
	Original Diet		Revised Diet		Control	Ethanol	Control	Ethanol
	Control	Ethanol	Control	Ethanol				
	C-C	E-E	*C-*C	*E-*E	*C-Ch	*E-Ch	Ch-*C	Ch-*E
	n=6		n=6		n=3		n=3	
10	91±31†	53±16†	N.A.	N.A.	220±7	201±43	191±8	211±33
17	172±59†	143±43†	374±124	423±134	376±92	554±109#	535±8	597±86
24	189±44†	206±55†	535±125	511±125	446±96	629±118	588±23	592±130

Each value represents the mean  $\pm$  the standard deviation. A # indicates that values from control and ethanol pups from the same group are different at  $p < 0.05$ . A † indicates that values from animals whose mothers consumed the original diets and from animals whose mothers consumed the revised diets are different at  $p < 0.05$ . N.A. indicates that data from these animals is not available. The abbreviations C & E, \*C & \*E, and Ch stand for the diets (original control and ethanol, revised control and ethanol, and chow) that the gestational mothers-nursing mothers consumed on a chronic basis prior to and during gestation. All mothers were allowed to eat chow ad libitum after the 3rd postnatal day.

radioactivity in the various SPM protein species was determined.

Figure 3 depicts the typical distribution of radioactivity among fucosylated SPM glycoproteins from cross-fostered 10 day-old control (\*C-Ch) and ethanol (\*E-Ch) pups. The schematic representation of a stained gel indicates the position of the major bands relative to the gel fractions used for the determination of radioactivity. Numerous minor bands were also seen in the low molecular weight region of the gel. However, no attempt was made to resolve all the minor bands of SPM glycoproteins in this region, because of the low incorporation of precursor into these bands.

Although each graph was obtained from data from one gel (one pair of animals), the selected graphs are typical of those obtained from 6 gels (6 pairs of animals) at each age regardless of the assignment of isotopes.

The graph in figure 4 represents the ratio of the (% dpm in a given fraction from an ethanol pup)/(% dpm in a given fraction from a control pup) from developing non-cross-fostered (E-E and C-C) offspring. This type of comparison is made because it facilitates the visualization of differences between control and ethanol pups. Since the SPM proteins from a control pup and an ethanol pup (labelled with [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ]-fucose respectively or vice-versa) were co-electrophoresed on a single gel, identical

Figure 3: Distribution of Radioactivity among Fucosylated SPM Glycoproteins from 10 day-old Control and Ethanol Pups.

The lower figure is a graphic representation of a typical gel loaded with SPM proteins from 10 day-old cross-fostered control (\*C-Ch) and ethanol (\*E-Ch) pups. Approximately 36,000 [ $^3\text{H}$ ] dpm and 3,000 [ $^{14}\text{C}$ ] dpm were recovered from this gel. The gel was sliced according to the major bands depicted in the schematic representation of this gel. (Note - the drawing is not drawn to scale). Based on the migration of the SPM proteins in comparison to proteins of known molecular weights, one would expect to find the SPM proteins ( $\text{Na}^+\text{-K}^+$ )- APTase, tublin and actin in fractions 4, 8 and 9, respectively. Fractions >13 are 2 mm sections of the gels. Fraction 1 is the top (high molecular weight end) of the gel.

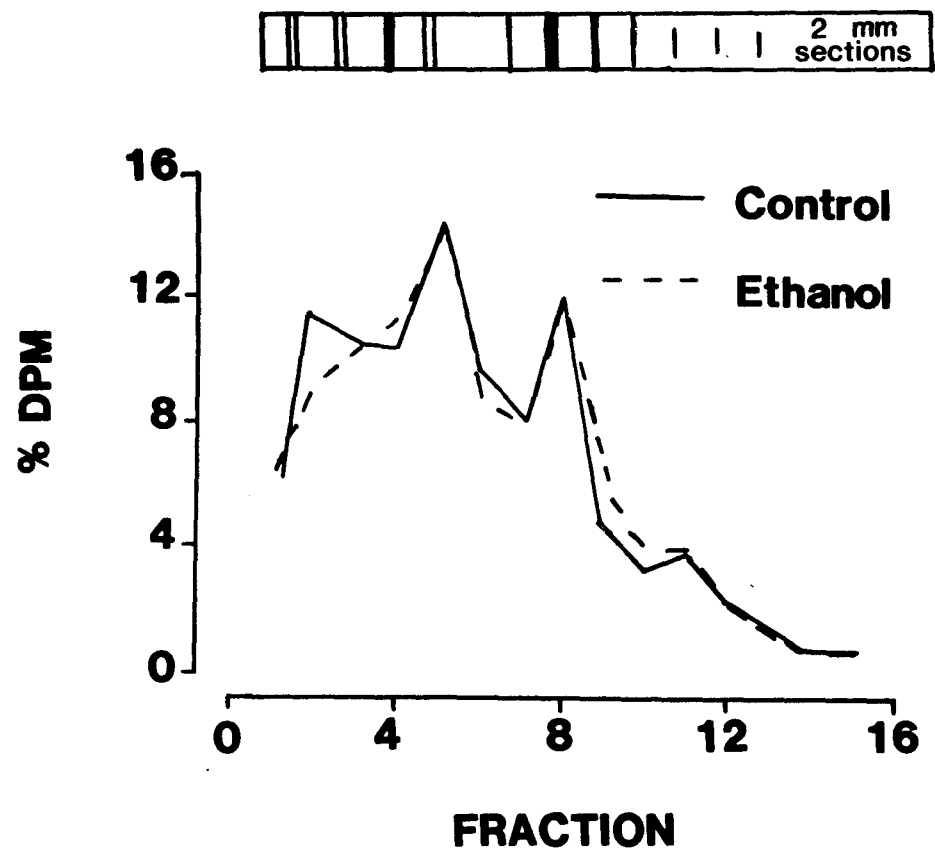
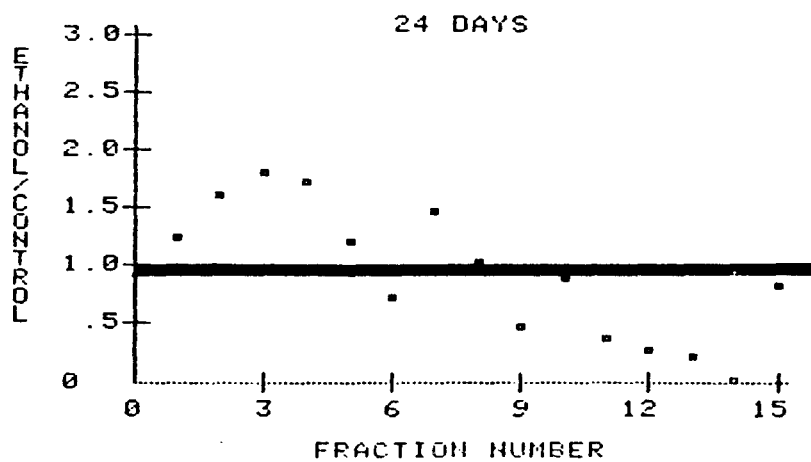
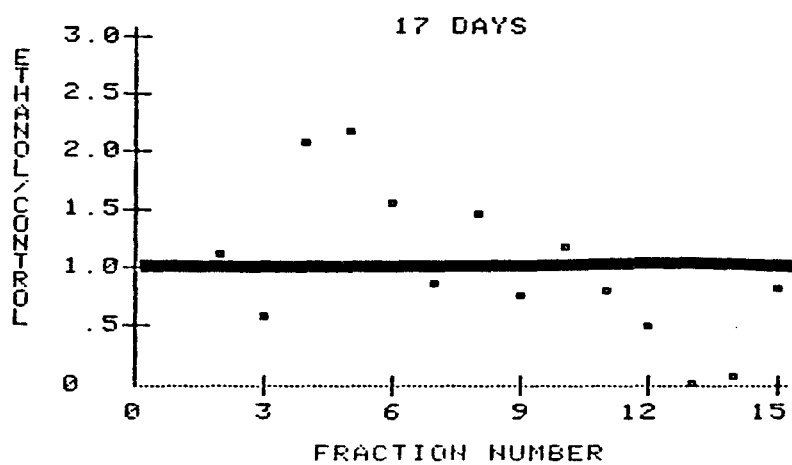
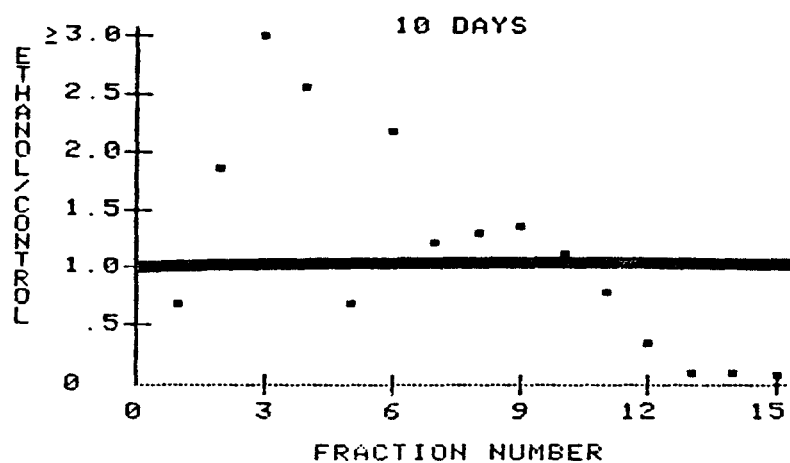


Figure 4: Ratios of (%dpm in a given fraction from an ethanol pup)/(%dpm in a given fraction from a control pup) from developing non-cross-fostered (E-E and C-C) rats. Greater than 4,000 [ $^{14}\text{C}$ ] dpm and 4,000-10,000 [ $^3\text{H}$ ]dpm were recovered from these gels. The assignment of isotopes did not influence the ratios.

Appendix Figures A-1 - A-3 graphically depict the distribution of radioactivity on the gels used in Figure 4.



proteins from the two animals should migrate to the same position on the gel. Thus, theoretically, if the post-natal synthesis of SPM glycoproteins is unaffected by prenatal ethanol exposure, one would expect a ratio of 1.0. In agreement with this hypothesis, ratios very close to 1.0 (ratios of 0.95 to 1.05) were observed by others in this laboratory in double-labelled studies of unaffected offspring of rat mothers that consumed ethanol only during the third trimester of gestation (figure 5, from Druse et al., 1981).

However, figure 4 demonstrates that chronic maternal ethanol consumption (original diet) results in abnormalities in the synthesis of SPM fucosylated glycoproteins in the non-cross-fostered offspring at all the ages examined. The abnormalities were more severe at the 10 day age period (note the large scale used to depict ethanol/control ratio in the figure).

In addition reasonably large ethanol/control ratios (less than 0.95 and greater than 1.05) were observed in the 10 day-old cross-fostered (\*E-Ch and \*C-Ch) offspring of rats that were fed the revised diet (figure 6). However, no large or consistent changes were seen in the older cross-fostered offspring (17 and 24 days) except in fractions 12-16 which contain the minor low molecular weight SPM proteins and hence have low radioactivity associated with them. Thus, it appears that chronic maternal ethanol

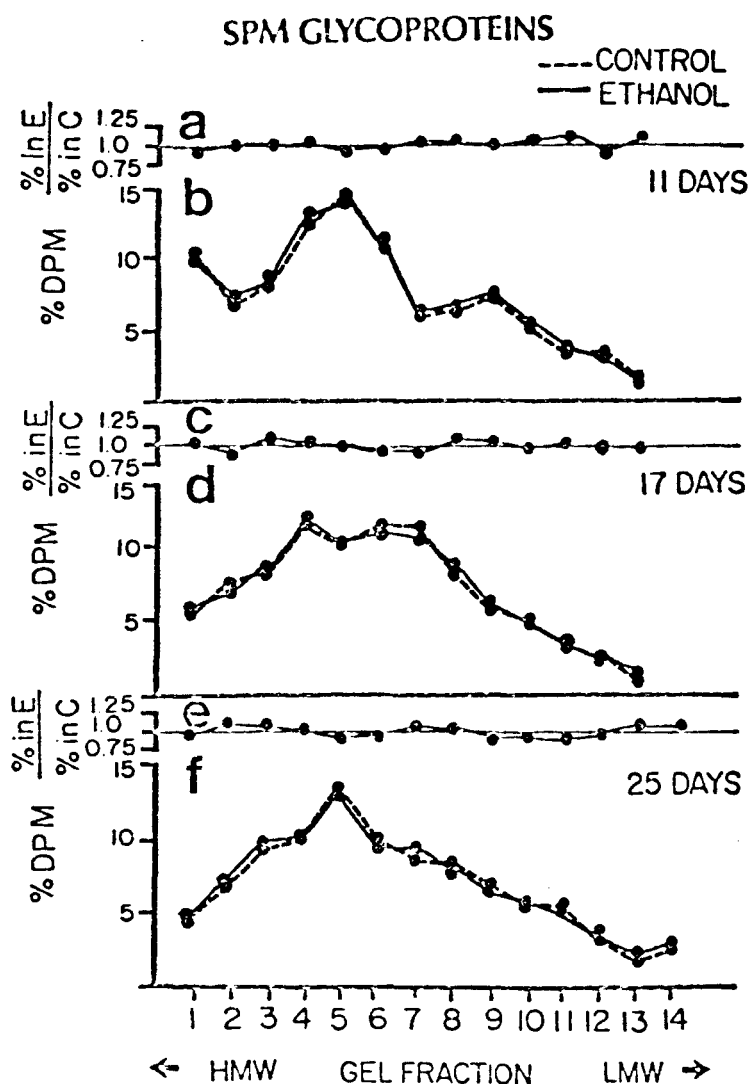


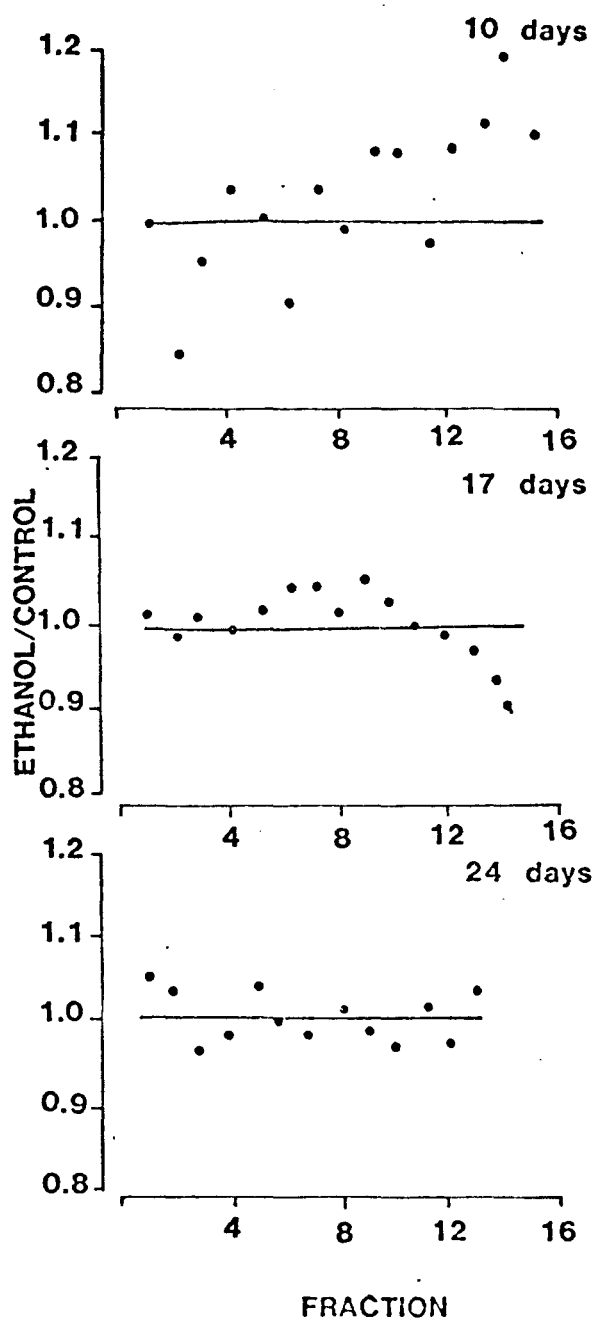
Figure 5

Synaptic Plasma Membrane Glycoproteins. The graphs in Figures b, d and f depict the relative distribution (% dpm) of the radioactivity solubilized from gels, loaded with a mixture of SPM proteins from control and ethanol pups. The graphs in a, c and e depict the ratios of the (% dpm in a given fraction from an ethanol rat)/(% dpm in a given fraction from a control rat). The SPM glycoproteins from control rats were labeled with L-[ $^3\text{H}$ ]-fucose, while those from the age-matched ethanol pups were labeled with L-[1- $^{14}\text{C}$ ]-fucose. The total radioactivity recovered from each gel ranged from 10,000 to 24,000 dpm for  $^3\text{H}$  and from 3,000 to 6,200 dpm for  $^{14}\text{C}$ . (from Druse *et al.*, 1981).



Figure 6: Ratios of (%dpm in a given fraction from an ethanol pup)/(%dpm in a given fraction from a control pup) from developing cross-fostered (\*E-Ch and \*C-Ch) rats. Greater than 36,000 [ $^3\text{H}$ ]dpm and 3,000-16,000 [ $^{14}\text{C}$ ]dpm were recovered from these gels. The assignment of isotopes did not influence the ratios.

Appendix figures A-4 - A-6 graphically depict the distribution of radioactivity on the gels used in Figure 6.



consumption results in abnormal synthesis of fucosylated SPM glycoproteins. SPM glycoprotein abnormalities are also present in rats that have been cross-fostered.

Figure 7 represents the ethanol/control ratios from developing reverse cross-fostered (Ch-\*E and Ch-\*C) rats. It appears that no large abnormalities are observed in the reverse cross-fostered animals except in the quantitatively minor fractions (greater than 12).

#### Microsomal, Synaptosomal and Synaptic Plasma Membrane Gangliosides

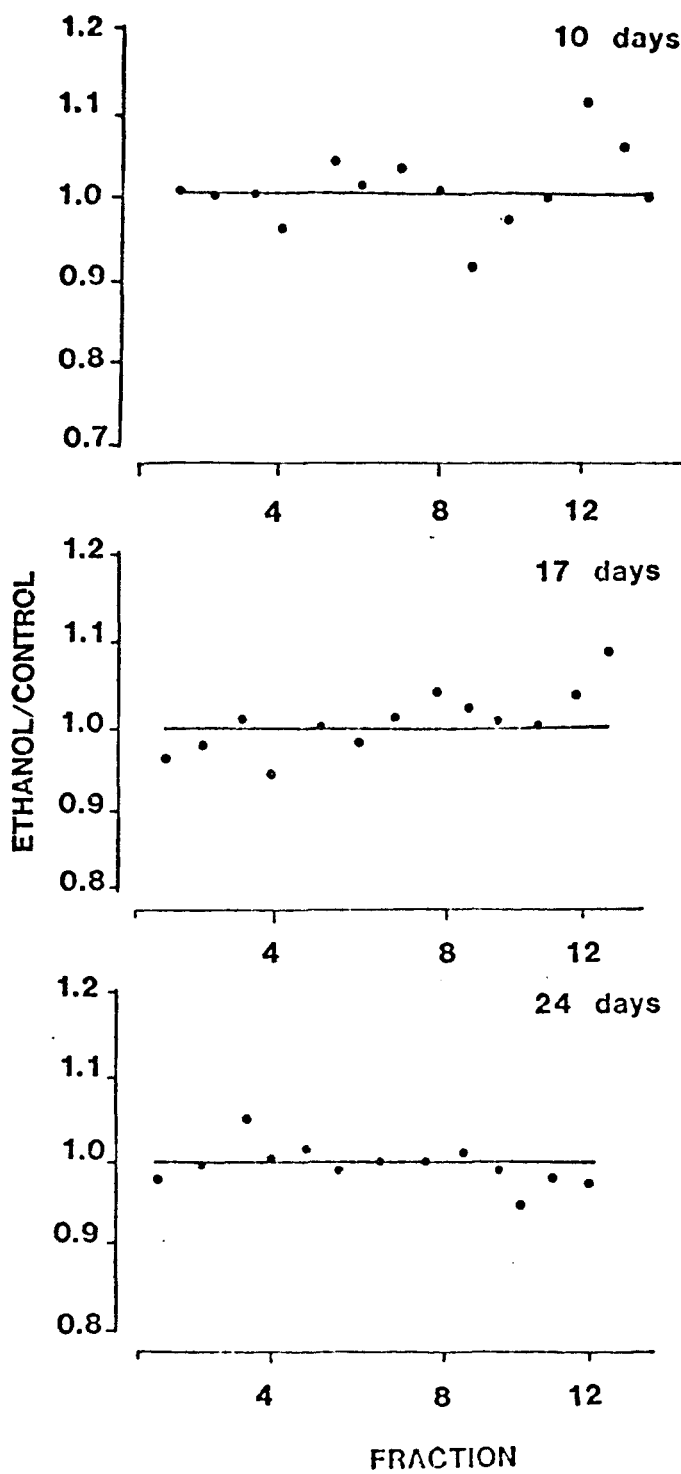
The gangliosides of three subcellular fractions -- microsomal, synaptosomal and synaptic plasma membrane fractions -- were studied in terms of concentration, relative distribution and incorporation of radioactivity into individual gangliosides. Furthermore, we examined the effects of chronic maternal ethanol consumption on the concentration and relative distribution of individual gangliosides in the mentioned three subcellular fractions.

In the present study and in all subsequent studies included in this Chapter the liquid diet utilized was the revised liquid diet \*C-\*C and \*E-\*E. As seen from the data presented in the previous section of this Chapter, the use of the revised liquid diet is preferable to that of the original liquid diet in terms of yield of SPMS.

The concentration of ganglioside sialic acid in

Figure 7: Ratios of (%dpm in a given fraction from an ethanol pup)/(%dpm in a given fraction from a control pup) from developing reverse cross-fostered (Ch-\*E and Ch-\*C) rats. Greater than 44,000 [ $^3\text{H}$ ] dpm and 7,000-16,000 [ $^{14}\text{C}$ ] dpm were recovered from these gels. The assignment of isotopes did not influence the ratios.

Appendix figures A-7 - A-9 graphically depict the distribution of radioactivity on the gels used in Figure 7.



microsomal membranes from ethanol and age-matched control pups was examined at 10 , 17 and 24 days of age (Table IX). Values for the sialic acid concentration of microsomal membranes are comparable to that reported by Yohe et al. (1980). No significant developmental differences were observed in the sialic acid concentration of microsomal membranes even though there seemed to be a moderate increase between 17 and 24 days. The ethanol pups consistently had a small (but not significant) decrease in the concentration of sialic acid.

Table X includes the sialic acid concentration of synaptosomal membranes from 10 to 24 day old ethanol and control pups. At each age, ganglioside sialic acid concentration of the synaptosomal membranes was approximately half that of the microsomal membranes. Similar observations have been noted by Yohe et al. (1980). The synaptosomal membranes also showed a moderate (but insignificant) age-related increase in sialic acid concentration that was similar to but less than that of the microsomal membranes. The concentration of synaptosomal ganglioside sialic acid was similar in ethanol and control pups.

The percent distribution of radiolabelled N-acetylmannosamine associated with individual microsomal and synaptosomal gangliosides was determined. At all the ages, GM<sub>1</sub>, GD<sub>1a</sub>, GD<sub>1b</sub> and GT<sub>1b</sub> were the most heavily labelled gangliosides in the microsomal membranes (figure 8, 9 and

TABLE IX

Effects of Maternal Ethanol Consumption  
on Sialic Acid Concentration of Microsomal Membranes in Offspring

---

$\mu\text{g}$ of Microsomal sialic acid per mg of protein		
Age in Days	Control	Ethanol
<hr/>		
10	16.5 $\pm$ 1.6	15.7 $\pm$ 3.3
17	20.9 $\pm$ 4.9	19.6 $\pm$ 2.3
24	20.6 $\pm$ 1.3	20.3 $\pm$ 1.8

---

Each value represents the mean  $\pm$  the standard deviation of three animals. Total ganglioside sialic acid was determined by Svennerholm's (1957) resorcinol method as modified by Miettinen and Takki-Luukkainen (1959).

TABLE X

Effects of Maternal Ethanol Consumption  
on Sialic Acid Concentration of Synaptosomal Membranes in Offspring

---

$\mu\text{g}$ of Synaptosomal sialic acid per mg of protein		
Age in Days	Control	Ethanol
<hr/>		
10	9.1 $\pm$ 1.0	9.1 $\pm$ 1.1
17	11.5 $\pm$ 1.2	11.1 $\pm$ 1.4
24	11.9 $\pm$ 1.2	12.2 $\pm$ 0.4

---

Each value represents the mean  $\pm$  the standard deviation of three animals. Total ganglioside sialic acid was determined by Svennerholm's (1957) resorcinol method as modified by Miettinen and Takki-Luukkainen (1959).



Figure 8 : Effects of Maternal Ethanol Consumption on the % distribution of Sialic Acid among Individual Radio-labeled Gangliosides of Microsomal Membranes at 10 days in Offspring.

Radiolabeled gangliosides were extracted from the microsomal membranes of individual control (C) and ethanol (E) pups at 10 days of age. A mixture of radiolabeled gangliosides from a control (C) and ethanol (E) rat of the same age was spotted on a HPTLC plate and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by exposing the plate to I<sub>2</sub> vapor in a glass tank. Individual gangliosides were scraped from the plate and solubilized in water and 10 ml of Aquasol. Radioactivity was determined by means of a liquid scintillation counter. Approximately 8,000 [<sup>14</sup>C] dpm (control) and 6,000 [<sup>14</sup>C] dpm (ethanol) were recovered from each lane. Results are expressed as the percentage of total DPM of ganglioside sialic acid DPM.

Each value represents the mean  $\pm$  the standard deviation of three animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

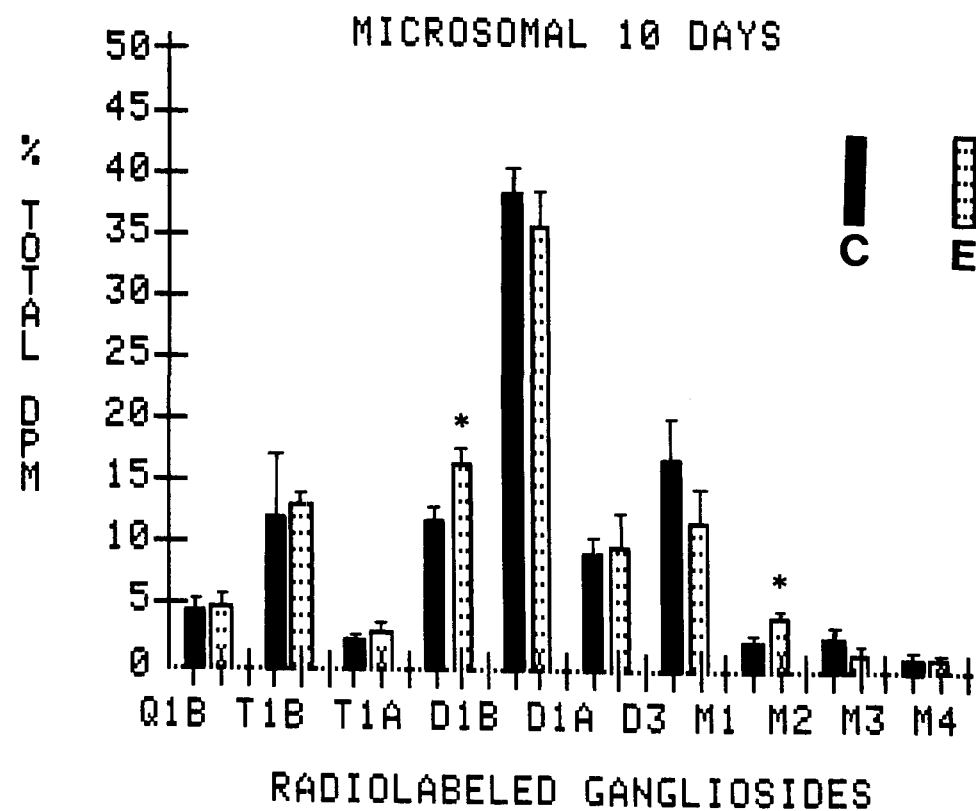


Figure 9 : Effects of Maternal Ethanol Consumption on the % distribution of Sialic Acid among Individual Radio-labeled Gangliosides of Microsomal Membranes at 17 days in Offspring.

Radiolabeled gangliosides were extracted from the microsomal membranes of individual control (C) and ethanol (E) pups at 17 days of age. A mixture of radiolabeled gangliosides from a control (C) and ethanol (E) rat of the same age was spotted on a HPTLC plate and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by exposing the plate to I<sub>2</sub> vapor in a glass tank. Individual gangliosides were scraped from the plate and solubilized in water and 10 ml of Aquasol. Radioactivity was determined by means of a liquid scintillation counter. Approximately 12,000 [<sup>14</sup>C] dpm (control) and 12,000 [<sup>14</sup>C] dpm (ethanol) were recovered from each lane. Results are expressed as the percentage of total DPM of ganglioside sialic acid DPM.

Each value represents the mean  $\pm$  the standard deviation of three animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

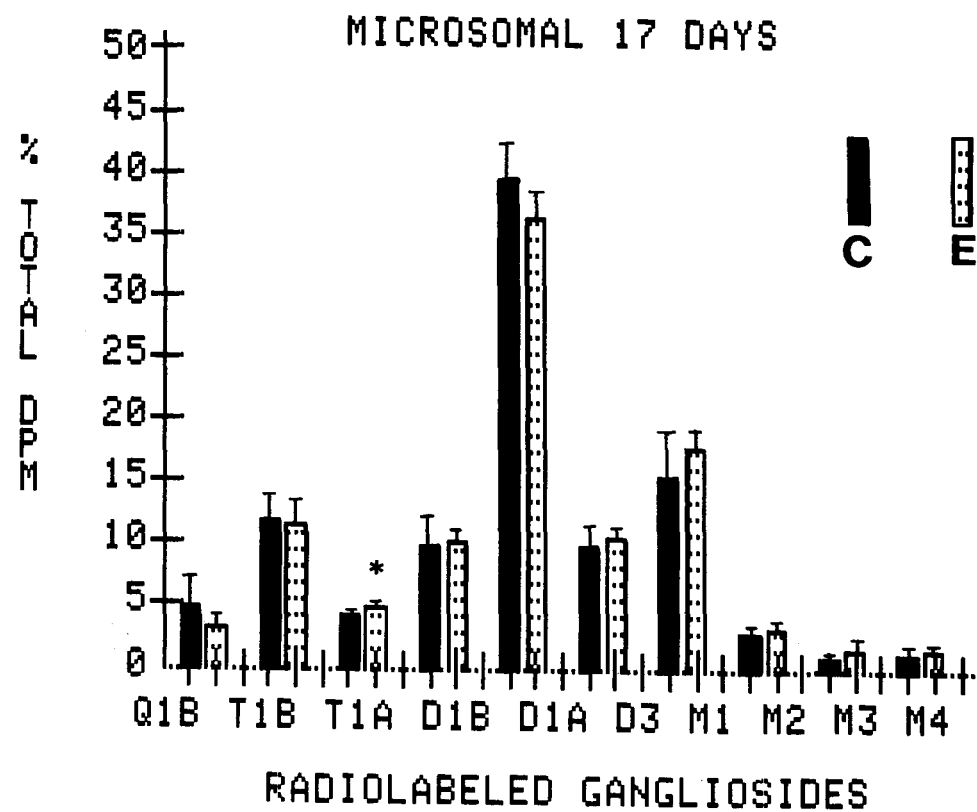
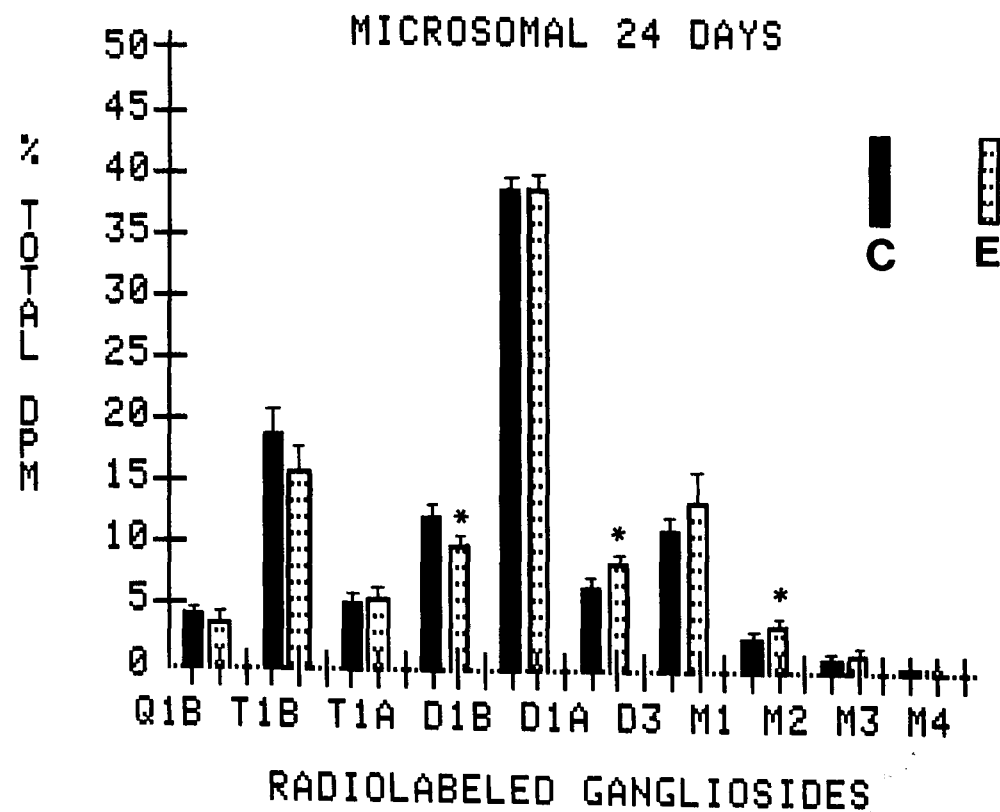


Figure 10: Effects of Maternal Ethanol Consumption on the % distribution of Sialic Acid among Individual Radiolabeled Gangliosides of Microsomal Membranes at 24 days in Offspring.

Radiolabeled gangliosides were extracted from the microsomal membranes of individual control (C) and ethanol (E) pups at 24 days of age. A mixture of radiolabeled gangliosides from a control (C) and ethanol (E) rat of the same age was spotted on a HPTLC plate and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by exposing the plate to I<sub>2</sub> vapor in a glass tank. Individual gangliosides were scraped from the plate and solubilized in water and 10 ml of Aquasol. Radioactivity was determined by means of a liquid scintillation counter. Approximately 10,000 [<sup>14</sup>C] dpm (control) and 12,000 [<sup>14</sup>C] dpm (ethanol) were recovered from each lane. Results are expressed as the percentage of total DPM of ganglioside sialic acid DPM.

Each value represents the mean  $\pm$  the standard deviation of three animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.



10).  $GD_{1a}$  consistently accounted for the greatest proportion of radioactivity (approximately 38-40%). Radioactivity was also detected in  $GD_3$ ,  $GT_{1a}$ ,  $GQ_{1b}$  and in the monosialogangliosides  $GM_2$ ,  $GM_3$  and  $GM_4$ . The percent distribution of radioactivity associated with each ganglioside in the microsomal membranes remained fairly constant throughout the ages examined.

Transient abnormalities were observed in the distribution of radioactivity among microsomal gangliosides in the ethanol pups. Ethanol pups showed a small but significant ( $p < 0.02$ ) increase in  $GT_{1a}$  at 17 days and  $GD_3$  at 24 days. Abnormalities were also observed in  $GD_{1b}$  and  $GM_2$  at 10 and 24 days.

Figures 11, 12 and 13 represent the percent distribution of radioactivity associated with individual synaptosomal gangliosides at 10, 17 and 24 days. Even though the synaptosomal membranes had approximately half the concentration of ganglioside sialic acid (Table X) as that of the microsomal membranes (Table IX), the percent distribution of radiolabelled gangliosides associated with the synaptosomal membranes was very similar to that of the microsomal membranes. As in the microsomal fraction, the greatest proportion of radioactivity in the synaptosomal membranes was associated with  $GT_1$ ,  $GD_{1b}$ ,  $GD_{1a}$  and  $GM_1$ .  $GD_{1a}$  consistently was the most heavily labelled synaptosomal ganglioside. However, unlike the microsomal fraction, negligible

Figure 11: Effects of Maternal Ethanol Consumption on the % distribution of Sialic Acid among Individual Radio-labeled Gangliosides of Synaptosomal Membranes at 10 days in Offspring.

Radiolabeled gangliosides were extracted from the synaptosomal membranes of individual control (C) and ethanol (E) pups at 10 days of age. A mixture of radiolabeled gangliosides from a control (C) and ethanol (E) rat of the same age was spotted on a HPTLC plate and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by exposing the plate to I<sub>2</sub> vapor in a glass tank. Individual gangliosides were scraped from the plate and solubilized in water and 10 ml of Aquasol. Radioactivity was determined by means of a liquid scintillation counter. Approximately 2,000 [<sup>14</sup>C] dpm (control) and 2,500 [<sup>14</sup>C] dpm (ethanol) were recovered from each lane. Results are expressed as the percentage of total DPM of ganglioside sialic acid DPM.

Each value represents the mean  $\pm$  the standard deviation of three animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.



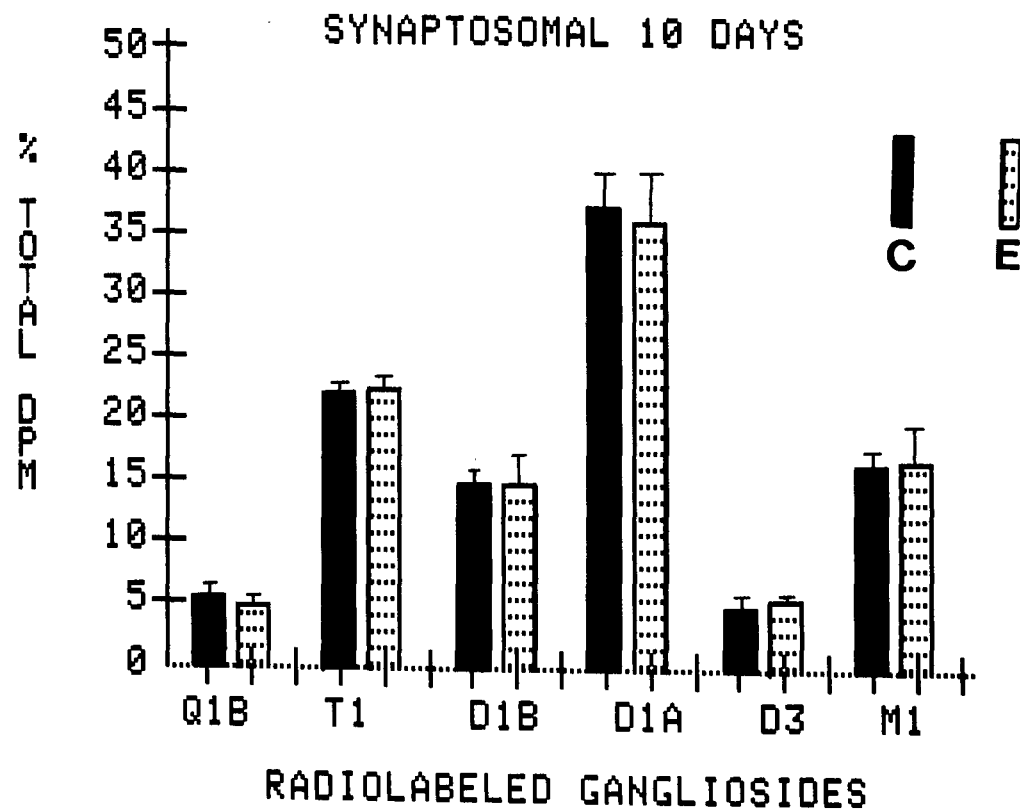


Figure 12: Effects of Maternal Ethanol Consumption on the % distribution of Sialic Acid among Individual Radiolabeled Gangliosides of Synaptosomal Membranes at 17 days in Offspring.

Radiolabeled gangliosides were extracted from the synaptosomal membranes of individual control (C) and ethanol (E) pups at 17 days of age. A mixture of radiolabeled gangliosides from a control (C) and ethanol (E) rat of the same age was spotted on a HPTLC plate and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by exposing the plate to I<sub>2</sub> vapor in a glass tank. Individual gangliosides were scraped from the plate and solubilized in water and 10 ml of Aquasol. Radioactivity was determined by means of a liquid scintillation counter. Approximately 3,500 [<sup>14</sup>C] dpm (control) and 3,500 [<sup>14</sup>C] dpm (ethanol) were recovered from each lane. Results are expressed as the percentage of total DPM of ganglioside sialic acid DPM.

Each value represents the mean  $\pm$  the standard deviation of three animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

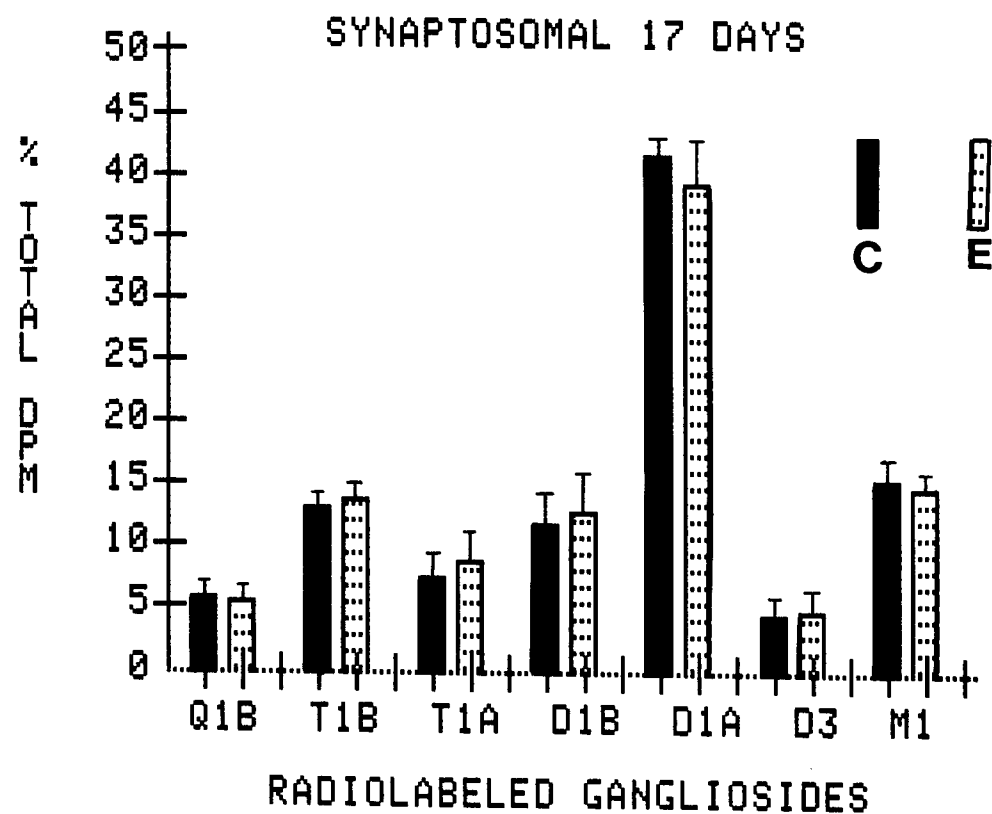
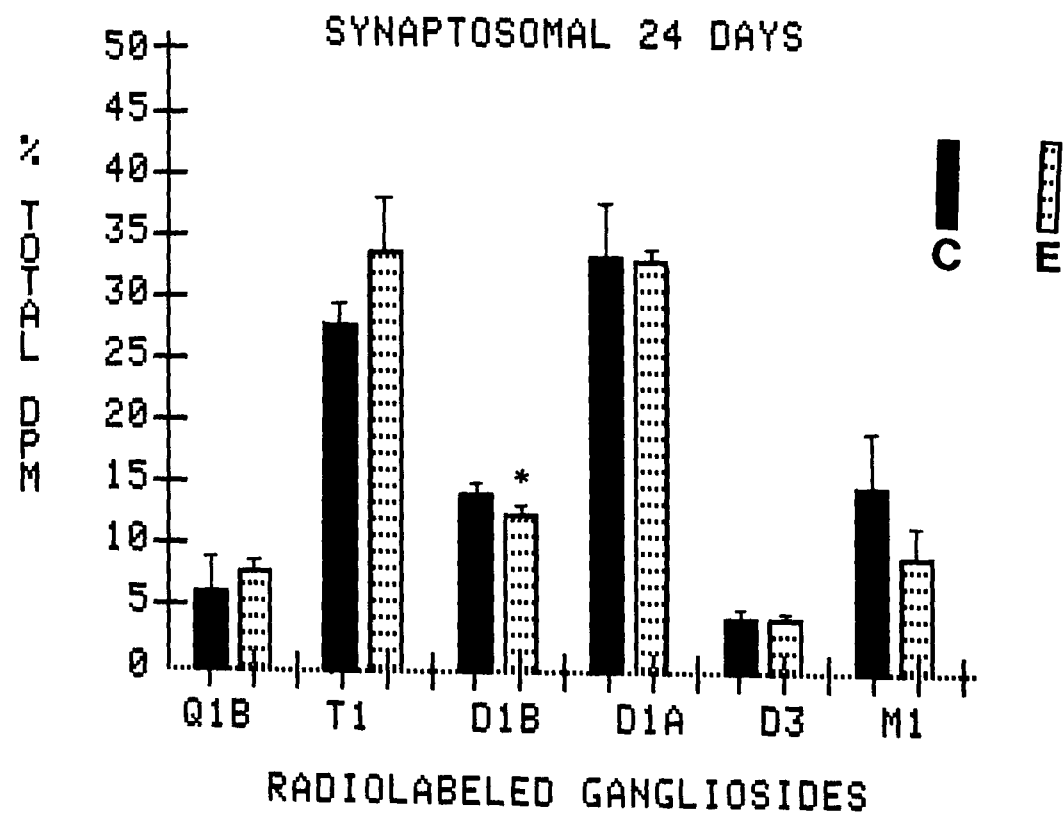


Figure 13: Effects of Maternal Ethanol Consumption on the % distribution of Sialic Acid among Individual Radiolabeled Gangliosides of Synaptosomal Membranes at 24 days in Offspring.

Radiolabeled gangliosides were extracted from the synaptosomal membranes of individual control (C) and ethanol (E) pups at 24 days of age. A mixture of radiolabeled gangliosides from a control (C) and ethanol (E) rat of the same age was spotted on a HPTLC plate and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by exposing the plate to I<sub>2</sub> vapor in a glass tank. Individual gangliosides were scraped from the plate and solubilized in water and 10 ml of Aquasol. Radioactivity was determined by means of a liquid scintillation counter. Approximately 5,000 [<sup>14</sup>C] dpm (control) and 6,000 [<sup>14</sup>C] dpm (ethanol) were recovered from each lane. Results are expressed as the percentage of total DPM of ganglioside sialic acid DPM.

Each value represents the mean  $\pm$  the standard deviation of three animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.



amounts of radioactivity were found associated with the monosialogangliosides GM<sub>2</sub>, GM<sub>3</sub> and GM<sub>4</sub>. Since the monosialoganglioside GM<sub>4</sub> is reportedly concentrated in myelin, this observation suggests a good separation of the synaptosomal and myelin membranes. The percent distribution of radioactivity associated with individual synaptosomal gangliosides appeared to be fairly constant over the age range examined. GM<sub>1</sub> showed a progressive (but insignificant) decrease with age.

The distribution of radioactivity among the synaptosomal gangliosides in ethanol and control pups were very similar. The only difference observed was a significant decrease ( $p < 0.02$ ) in GD<sub>1b</sub> in the ethanol pups at 24 days of age.

#### Synaptic Plasma Membrane Gangliosides

The concentration of ganglioside sialic acid in synaptic plasma membranes at 17 , 24 and 31 days of age are tabulated in Table XI. At all the ages examined, the concentration of ganglioside sialic acid was over 20  $\mu$ g per mg of SPM protein in both control and ethanol pups. This high concentration of ganglioside sialic acid in SPM clearly demonstrates the enrichment of gangliosides in nerve endings.

The sialic acid concentration of SPMs seemed to be fairly constant at all the age periods studied. Even though the ethanol pups consistently demonstrated an increased concentration of ganglioside sialic acid it was not

TABLE XI

Effects of Maternal Ethanol Consumption  
on Sialic Acid Concentration of Synaptic Plasma Membranes in Offspring

---

$\mu\text{g}$ of SPM sialic acid per mg of protein		
Age in Days	Control	Ethanol
<hr/>		
17	20.6 $\pm$ 5.1	23.9 $\pm$ 10.8
24	21.5 $\pm$ 3.4	21.9 $\pm$ 1.7
31	24.7 $\pm$ 4.2	26.7 $\pm$ 2.9

---

Each value represents the mean  $\pm$  the standard deviation of six animals. Total ganglioside sialic acid was determined by Svennerholm's (1957) resorcinol method as modified by Miettinen and Takki-Luukkainen (1959).

statistically significant.

Figure 14 depicts a thin-layer chromatogram of SPM gangliosides and the densitometric scan of the gangliosides. The percent distribution of the peak area was calculated directly by means of a Hewlett Packard 3390A integrator which was coupled to the densitometer. With a typical thin-layer chromatogram, about 98% of the total peak areas determined by the integrator are associated with the gangliosides shown in figure 14. Accordingly, the density distributions were corrected for a total density of 100%.

Figures 15, 16 and 17 represent the corrected percent distribution of sialic acid among individual SPM gangliosides at the three age periods studied. At all the ages examined GD<sub>1a</sub> and GT<sub>1b</sub> were the predominant gangliosides and accounted for approximately 26-35% and 23-28%, respectively, of the total SPM ganglioside sialic acid in the control pups. As in the microsomal and synaptosomal membranes, SPM also contained GQ<sub>1b</sub>, GT<sub>1a</sub>, GD<sub>1b</sub>, GD<sub>3</sub> and GM<sub>1</sub>.

No striking developmentally related changes were observed in the relative proportions of the individual gangliosides of SPMs. When control and ethanol pups were compared, ethanol pups had a significantly ( $p < 0.02$ ) higher level of GD<sub>1a</sub> at 17 days (figure 15). No significant differences were observed in the relative proportions of the other SPM gangliosides at 24 days of age.



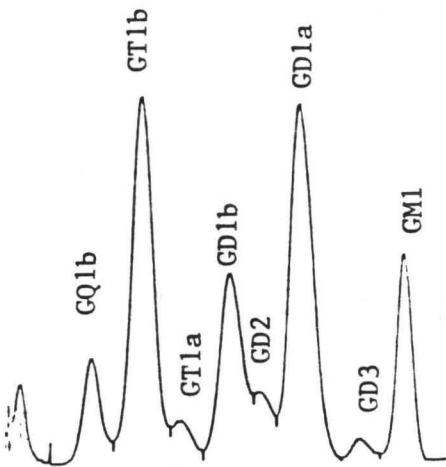
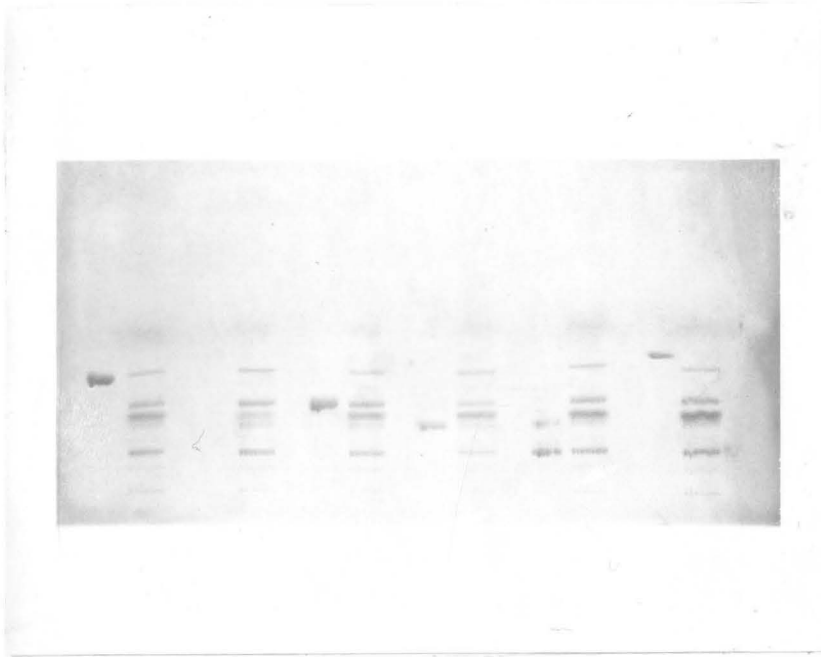


Figure 14: Thin-layer chromatogram of SPM ganglioside samples of control (C) and ethanol (E) rats at 17 days of age. SPM ganglioside sialic acid was spotted as a 1.5 cm streak and the plate was developed in Methyl Acetate/propanol/chloroform/methanol/aqueous 0.25% KCl (25:20:20:20:17 v/v). Gangliosides were visualized by resorcinol spray. The densitometric response of a single lane is shown by the pen tracing. The gangliosides are designated according to the nomenclature of Svernerholm (1963). The gangliosides accounted for nearly all the density calculated by the integrator.

Figure 15 : Effects of Maternal Ethanol Consumption on the % Distribution of Sialic Acid among Individual Gangliosides of SPM at 17 days of Age in Offspring.

Gangliosides were extracted from the SPMs of individual control (C) and ethanol (E) pups as described in "Materials and Methods" at 17 days of age. An aliquot of the extracted ganglioside sialic acid was spotted as a 1.5 cm streak on HPTLC plates (10X10 cm) and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by the resorcinol spray (Svennerholm, 1957). Identification of the ganglioside bands was achieved by comparing their chromatographic mobilities with the standards. The distribution of individual gangliosides was determined directly by densitometric scanning of the resorcinol positive spots, by the procedure of Ando et al. (1978). The percent distribution of individual gangliosides in each sample was determined by a 3390A integrator (Hewlett-Packard). Results are expressed as the percentage of total ganglioside sialic acid. Gangliosides are named according to SVENNERHOLM's nomenclature.

Each value represents the mean  $\pm$  the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

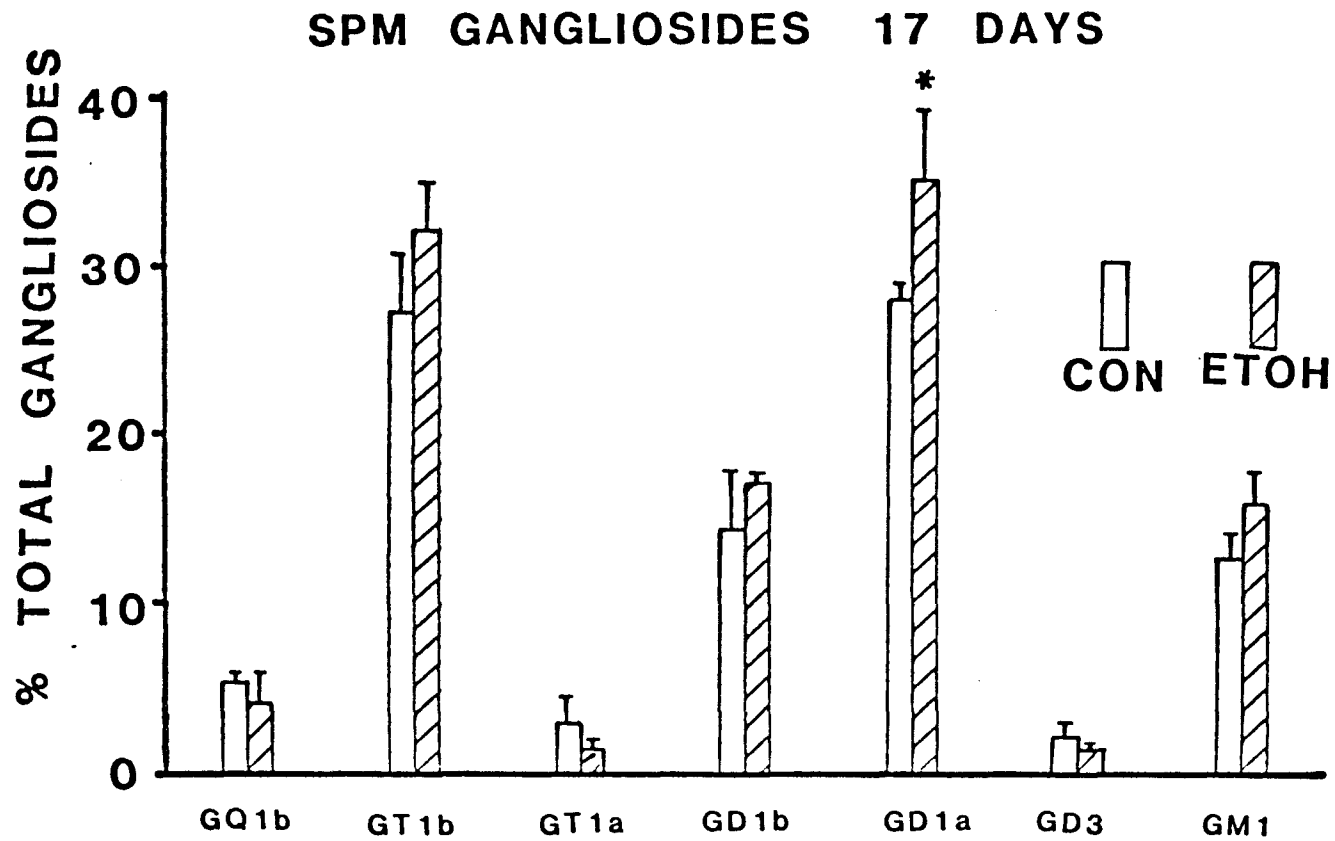


Figure 16 : Effects of Maternal Ethanol Consumption on the % Distribution of Sialic Acid among Individual Gangliosides of SPM at 24 days of Age in Offspring.

Gangliosides were extracted from the SPMs of individual control (C) and ethanol (E) pups as described in "Materials and Methods" at 24 days of age. An aliquot of the extracted ganglioside sialic acid was spotted as a 1.5 cm streak on HPTLC plates (10X10 cm) and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by the resorcinol spray (Svennerholm, 1957). Identification of the ganglioside bands was achieved by comparing their chromatographic mobilities with the standards. The distribution of individual gangliosides was determined directly by densitometric scanning of the resorcinol positive spots, by the procedure of Ando et al. (1978). The percent distribution of individual gangliosides in each sample was determined by a 3390A integrator (Hewlett-Packard). Results are expressed as the percentage of total ganglioside sialic acid. Gangliosides are named according to SVENNERHOLM's nomenclature.

Each value represents the mean  $\pm$  the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

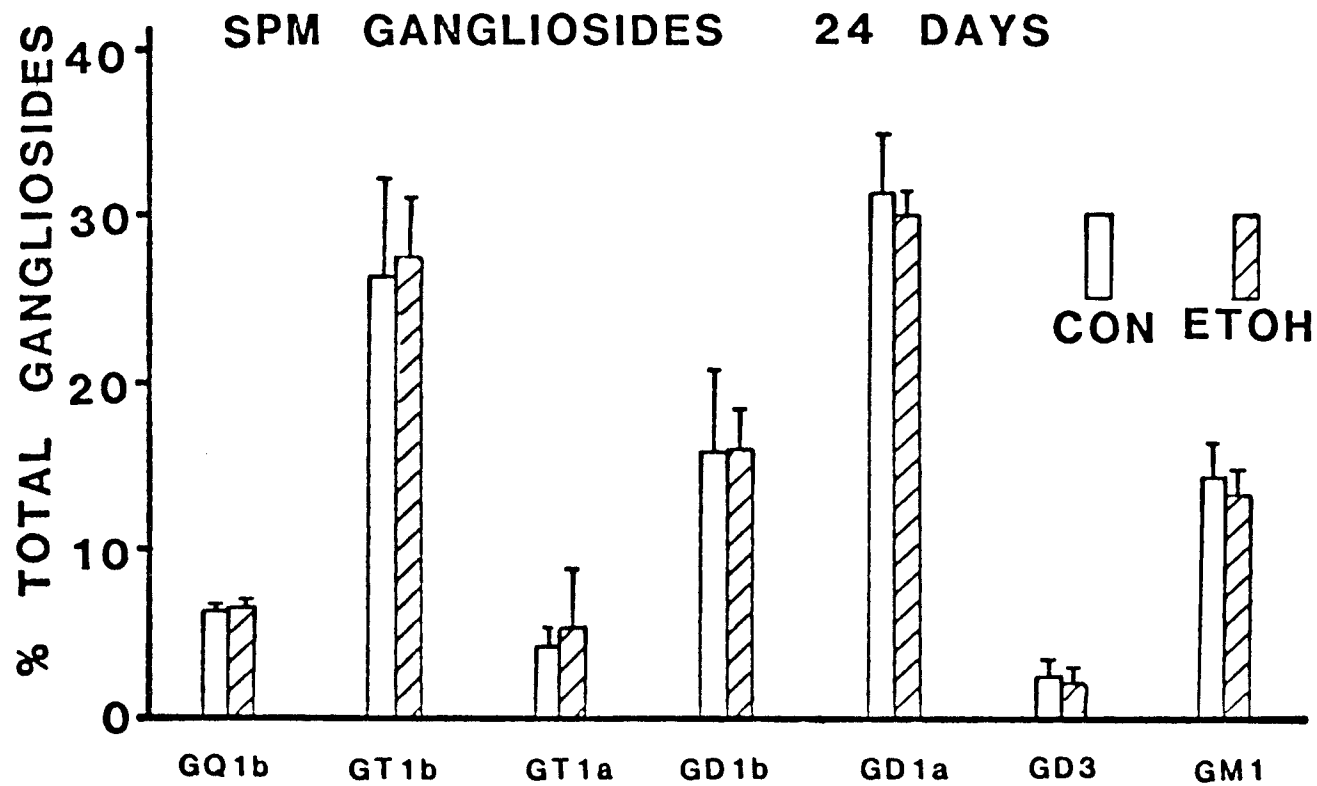
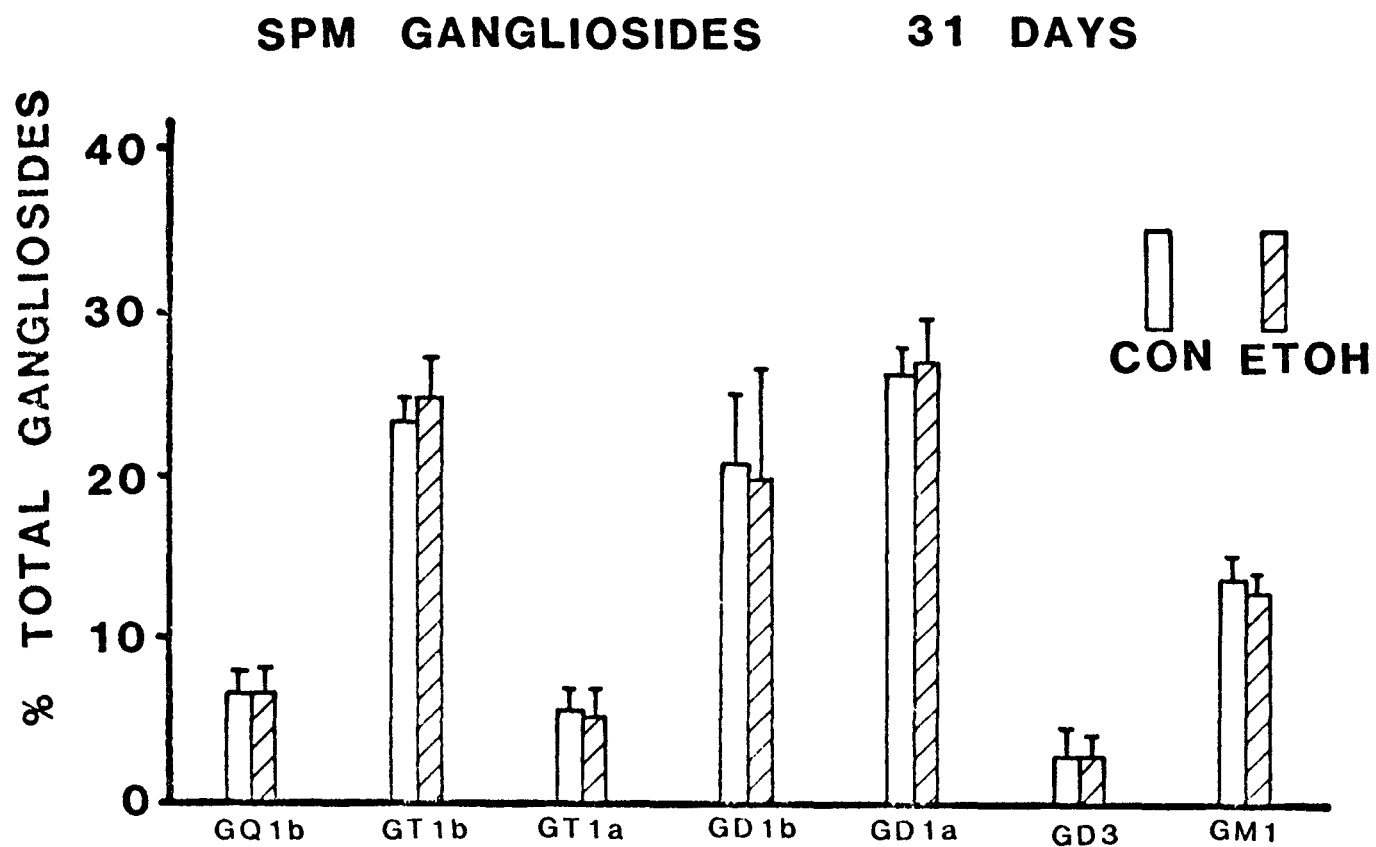


Figure 17 : Effects of Maternal Ethanol Consumption on the % Distribution of Sialic Acid among Individual Gangliosides of SPM at 31 days of Age in Offspring.

Gangliosides were extracted from the SPMs of individual control (C) and ethanol (E) pups as described in "Materials and Methods" at 31 days of age. An aliquot of the extracted ganglioside sialic acid was spotted as a 1.5 cm streak on HPTLC plates (10X10 cm) and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by the resorcinol spray (Svennerholm, 1957). Identification of the ganglioside bands was achieved by comparing their chromatographic mobilities with the standards. The distribution of individual gangliosides was determined directly by densitometric scanning of the resorcinol positive spots, by the procedure of Ando et al. (1978). The percent distribution of individual gangliosides in each sample was determined by a 3390A integrator (Hewlett-Packard). Results are expressed as the percentage of total ganglioside sialic acid. Gangliosides are named according to SVENNERHOLM's nomenclature.

Each value represents the mean  $\pm$  the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.



Figures 18, 19 and 20 show the percent distribution of radioactivity associated with the sialic acid of individual gangliosides of SPM between 17 and 31 days of age. At all ages, the major proportion of radioactivity was found associated with  $GD_{1a}$  and  $GT_{1b}$ .  $GD_{1a}$  accounted for approximately 23-30% of the total ganglioside sialic acid radioactivity while  $GT_{1b}$  accounted for approximately 28-34% of the radioactivity in the control pups.  $GM_2$  and  $GM_3$  accounted for 1% and 2% of the total ganglioside sialic acid radioactivity. It should be noted that these minor gangliosides could not be clearly visualized and quantitated when the chromatograms were sprayed with resorcinol-HCl reagent and densitometrically scanned.

At all ages, percent distribution of radioactivity associated with the sialic acid of each individual ganglioside (figures 18-20) correlated in most cases with the percent distribution of ganglioside sialic acid obtained from densitometric scans (figures 15-17). These results indicate that many of the gangliosides of the synaptic plasma membrane appear to be synthesized in a constant relation to its given concentration.

No significant developmentally related changes were observed in the proportion of radioactivity associated with individual gangliosides of synaptic plasma membranes. In addition, the proportion of the radioactivity incorporated into individual gangliosides of control and ethanol pups



Figure 18: Effects of Maternal Ethanol Consumption on the % Distribution of Sialic Acid among Individual Radiolabeled Gangliosides of SPM at 17 days in Offspring.

Radiolabeled gangliosides were extracted from the SPMs of individual control (C) and Ethanol (E) pups at 17 days of age. A mixture of radiolabeled gangliosides from a control (C) and ethanol (E) rat of the same age was spotted on a HPTLC plate and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by exposing the plate to I<sub>2</sub> vapor in a glass tank. Individual gangliosides were scraped from the plate and solubilized in water and 10 ml of Aquasol. Radioactivity was determined by means of a liquid scintillation counter. Approximately 1,700 [<sup>3</sup>H]dpm (control) and 350 [<sup>14</sup>C] dpm (ethanol) were recovered from each lane. Results are expressed as the percentage of total DPM of ganglioside sialic acid DPM.

Each value represents the mean  $\pm$  the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

# SPM RADIOLABELED GANGLIOSIDES

17 DAYS

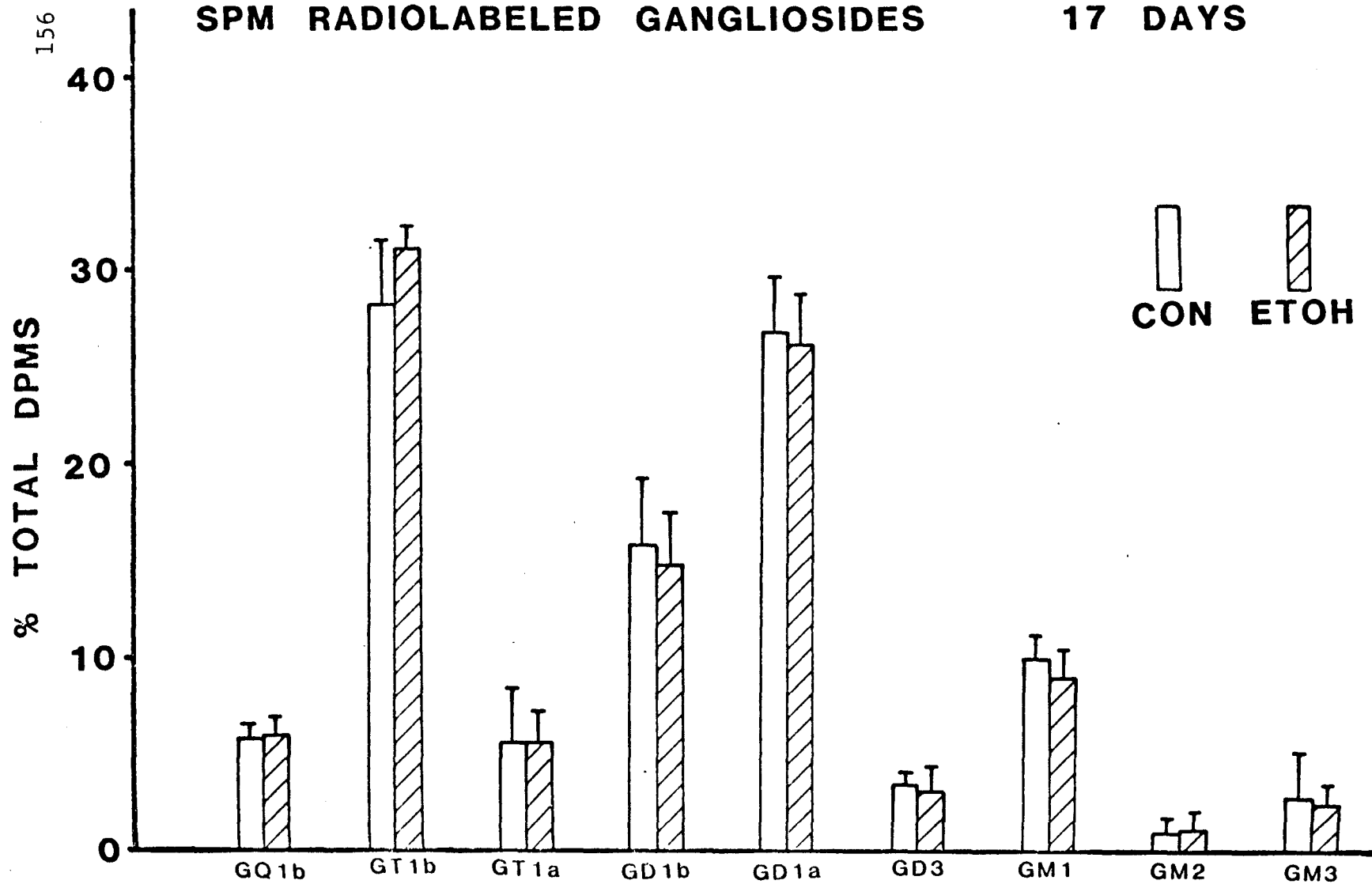


Figure 19: Effects of Maternal Ethanol Consumption on the % Distribution of Sialic Acid among Individual Radiolabeled Gangliosides of SPM at 24 days in Offspring.

Radiolabeled gangliosides were extracted from the SPMs of individual control (C) and ethanol (E) pups at 24 days of age. A mixture of radiolabeled gangliosides from a control (C) and ethanol (E) rat of the same age was spotted on a HPTLC plate and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by exposing the plate to I<sub>2</sub> vapor in a glass tank. Individual gangliosides were scraped from the plate and solubilized in water and 10 ml of Aquasol. Radioactivity was determined by means of a liquid scintillation counter. Approximately 2,900 [<sup>3</sup>H]dpm (control) and 700 [<sup>14</sup>C] dpm (ethanol) were recovered from each lane. Results are expressed as the percentage of total DPM of ganglioside sialic acid DPM.

Each value represents the mean  $\pm$  the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

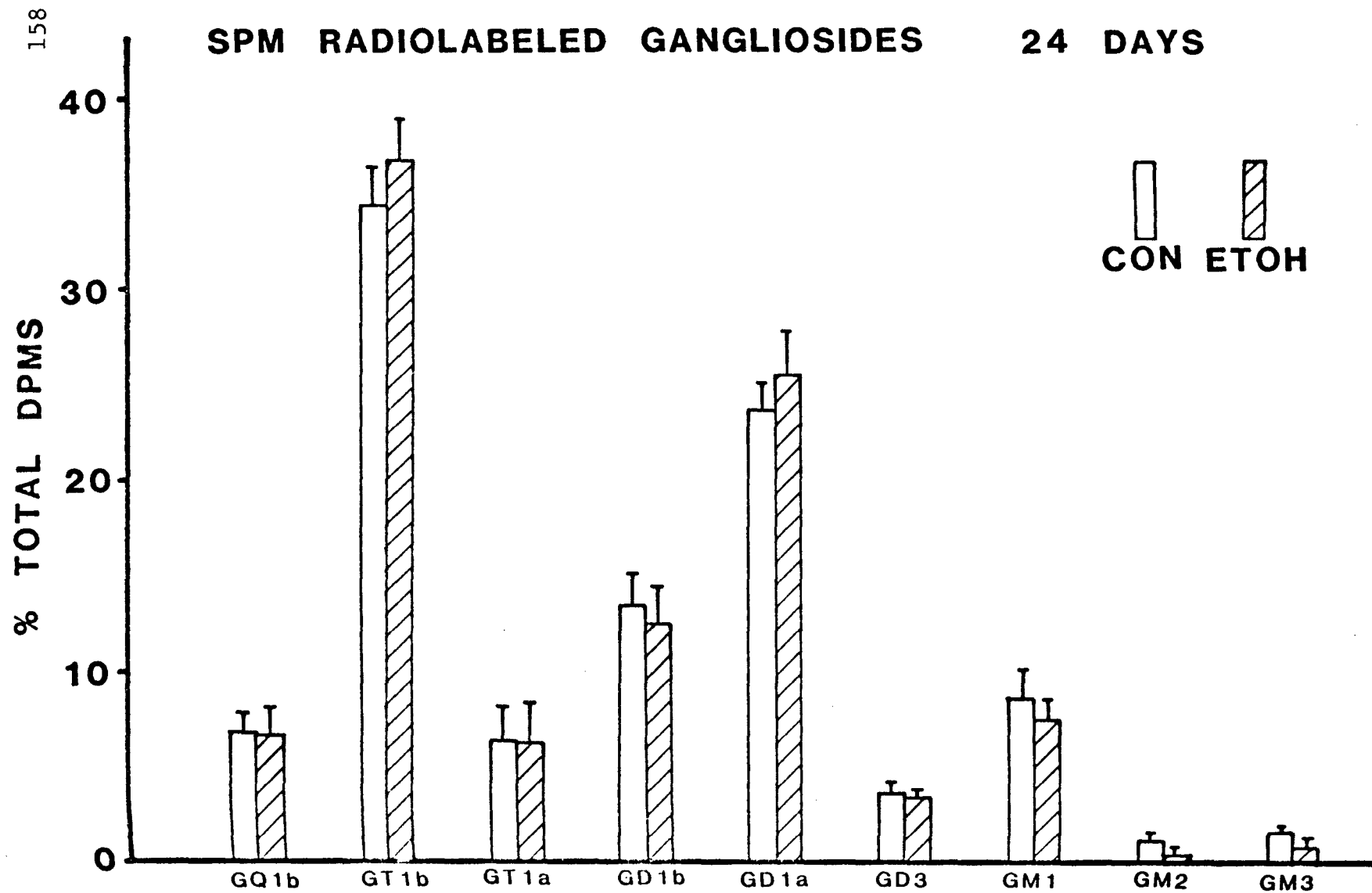


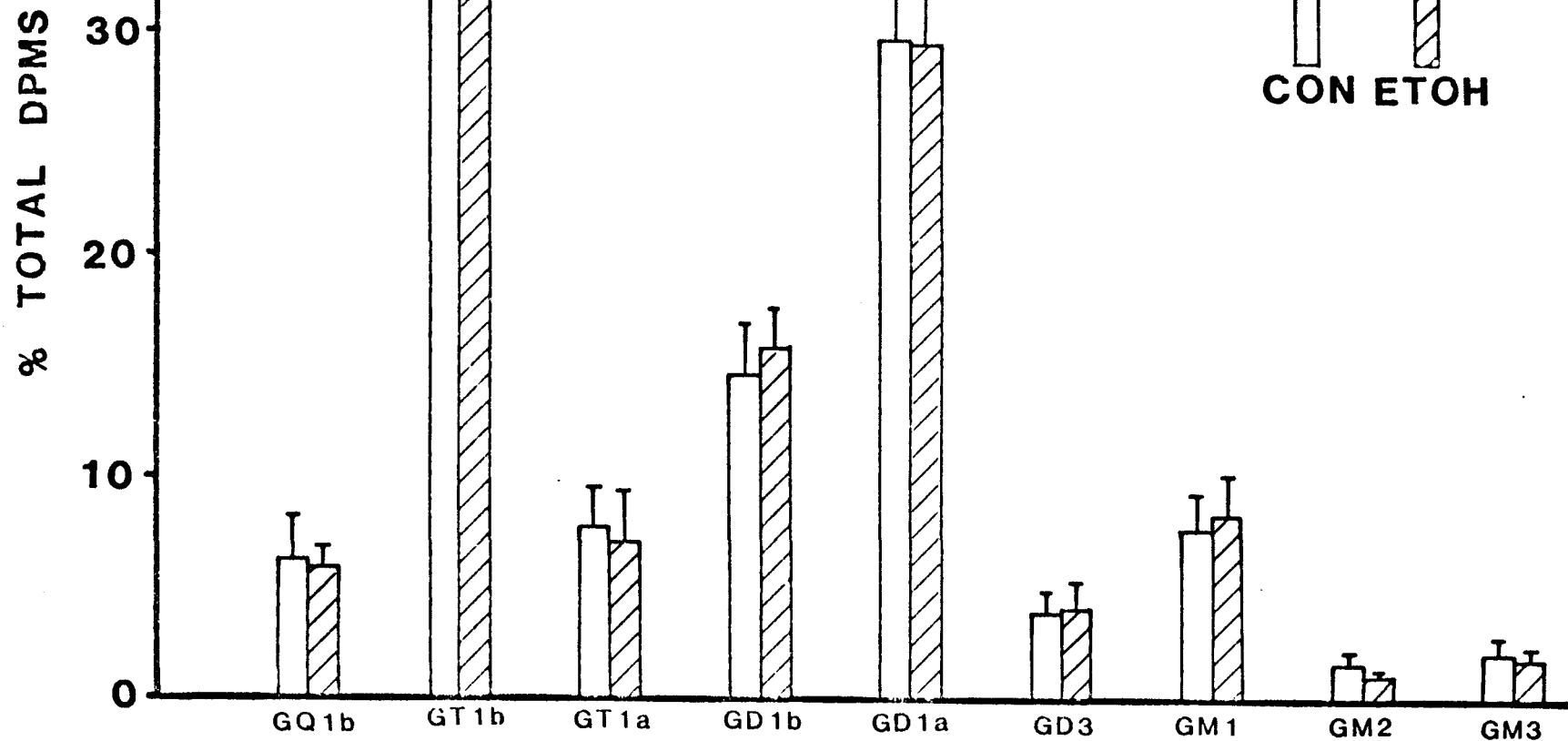
Figure 20 : Effects of Maternal Ethanol Consumption on the % Distribution of Sialic Acid among Individual Radiolabeled Gangliosides of SPM at 31 days in Offspring.

Radiolabeled gangliosides were extracted from the SPMs of individual control (C) and ethanol (E) pups at 31 days of age. A mixture of radiolabeled gangliosides from a control (C) and ethanol (E) rat of the same age was spotted on a HPTLC plate and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by exposing the plate to  $I_2$  vapor in a glass tank. Individual gangliosides were scraped from the plate and solubilized in water and 10 ml of Aquasol. Radioactivity was determined by means of a liquid scintillation counter. Approximately 7,700 [ $^3H$ ]dpm (control) and 650 [ $^{14}C$ ] dpm (ethanol) were recovered from each lane. Results are expressed as the percentage of total DPM of ganglioside sialic acid DPM.

Each value represents the mean  $\pm$  the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

## SPM RADIOLABELED GANGLIOSIDES 31 DAYS



similar at all ages and no significant differences were observed.

### Brain Regional Gangliosides

The effect of maternal ethanol consumption on the synthesis, distribution and concentration of gangliosides in six different brain regions of the offspring was examined at 10, 17 and 24 days of age. The brain regions included in this study were the cerebral cortex, cerebellum, hippocampus, hypothalamus, brain stem and the corpus striatum.

The liquid diet used for this study was the revised liquid diet, \*C-\*C and \*E-\*E.

Tables A-I - A-VI of the Appendix include the present distribution of sialic acid among the individual gangliosides of the different brain regions studies. The mean values and the standard deviations included in these tables were calculated from the densitometric scans of thin-layer chromatograms. The tables also show comparisons between ethanol and matched control pups.

The major gangliosides in all of the brain regions examined were  $GT_{1b}$  and  $GD_{1a}$ . Other gangliosides observed were  $GQ_{1b}$ ,  $GT_{1a}$ ,  $GD_{1b}$ ,  $GD_2$ ,  $GD_3$  and  $GM_1$ . In addition, measurable levels (>5% of total % distribution of gangliosides) of the monosialogangliosides  $GM_3$  and  $GM_4$  were seen in the cerebellum and the brain stem. These gangliosides were also observed in some of the other brain

regions but they were present in trace amounts.

### Development

Several trends were observed in the distribution of the various gangliosides with development. These developmental trends in the normal or control pups are commented upon below.

#### GQ<sub>1b</sub>

The proportion of the polysialoganglioside GQ<sub>1b</sub> showed a significant decrease ( $p < 0.005$ ) in the hippocampus and cerebral cortex between 10 and 24 days and in the brain stem between 10 and 17 days of age. (figure 21). However, in the cerebellum and corpus striatum the proportion of GQ<sub>1b</sub> seemed to remain fairly constant throughout development. Between the 17 day and 24 days of age, GQ<sub>1b</sub> showed a significant increase ( $p < 0.005$ ) in the hypothalamus. The hippocampus showed the highest proportion of GQ<sub>1b</sub> (>10% of the total gangliosides) at the early age period (10-days); At the same age period the cerebral cortex had the lowest proportion (<5%) when compared with the other brain regions.

#### GT<sub>1b</sub>

GT<sub>1b</sub> was one of the major ganglioside species observed in all the brain regions examined. The proportion



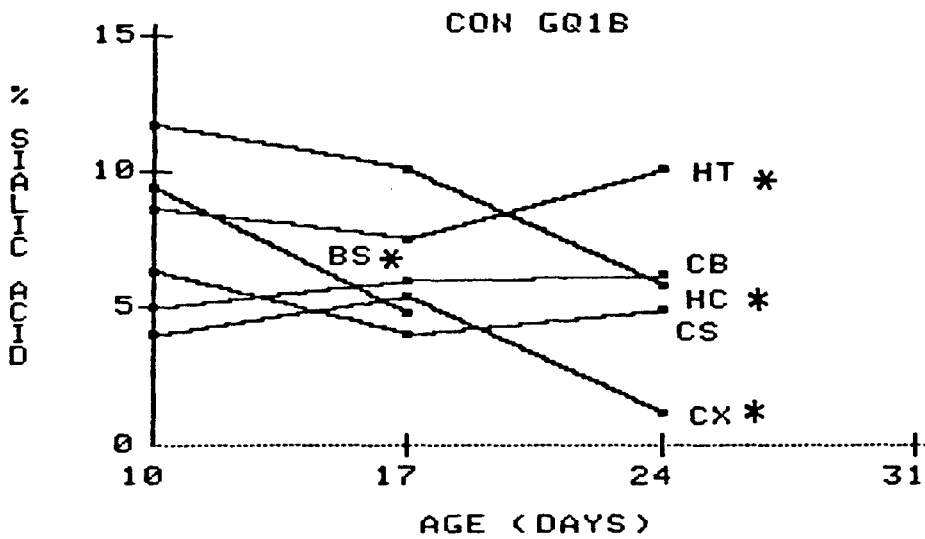
Figure 21: Developmental changes of GQ<sub>1b</sub> in selected brain regions of control (C) rats.

The figure depicts percent distribution (% total ganglioside sialic acid) of ganglioside GQ<sub>1b</sub> in the cerebral cortex (CX), cerebellum (CB), brain stem (BS), corpus striatum (CS), hypothalamus (HT) and hippocampus (HC) of control (C) (normal) rats with development. The percent distribution of GQ<sub>1b</sub> was determined by densitometric scanning of GQ<sub>1b</sub> on tlc plates.

Each point represents the mean value of six rats.

A \* indicates that there was a statistically significant ( $p < 0.05$ ) change in the percent distribution of the ganglioside during development in a particular brain region.

The standard deviations of each point are included in Tables A-I - A-VI of the Appendix.



of  $GT_{1b}$  showed a significant decrease ( $p < 0.005$ ) in the cerebral cortex and the cerebellum with development and between 17 and 24 days of age in the hippocampus (figure 22). On the other hand, the hypothalamus and the corpus striatum did not show any change during the age period examined.

#### $GT_{1a}$

$GT_{1a}$  appeared to be one of the minor gangliosides in all the brain regions. The proportion of  $GT_{1a}$  increased more than two-fold in the cerebellum and in the brain stem during this study (figure 23). This increase was statistically significant ( $p < 0.02$ ). However, the other brain regions had a constant proportion of  $GT_{1a}$  throughout the age period studied.

#### $GD_{1b}$

The proportion of this ganglioside appeared to be fairly constant throughout development in most of the brain regions (figure 24). The exception being a significant increase ( $p < 0.02$ ) (approximate two-fold increase) between 10 and 17 days in the brain stem. The hypothalamus also showed a significant increase ( $p < 0.005$ ) between 10 and 17 days.

Figure 22: Developmental changes of GT<sub>1b</sub> in selected brain regions of control (C) rats.

The figure depicts percent distribution (% total ganglioside sialic acid) of ganglioside GT<sub>1b</sub> in the cerebral cortex (CX), cerebellum (CB), brain stem (BS), corpus striatum (CS), hypothalamus (HT) and hippocampus (HC) of control (C) (normal) rats with development. The percent distribution of GT<sub>1b</sub> was determined by densitometric scanning of GT<sub>1b</sub> on tlc plates.

Each point represents the mean value of six rats.

A \* indicates that there was a statistically significant ( $p < 0.05$ ) change in the percent distribution of the ganglioside during development in a particular brain region.

The standard deviations of each point are included in Tables A-I - A-VI of the Appendix.

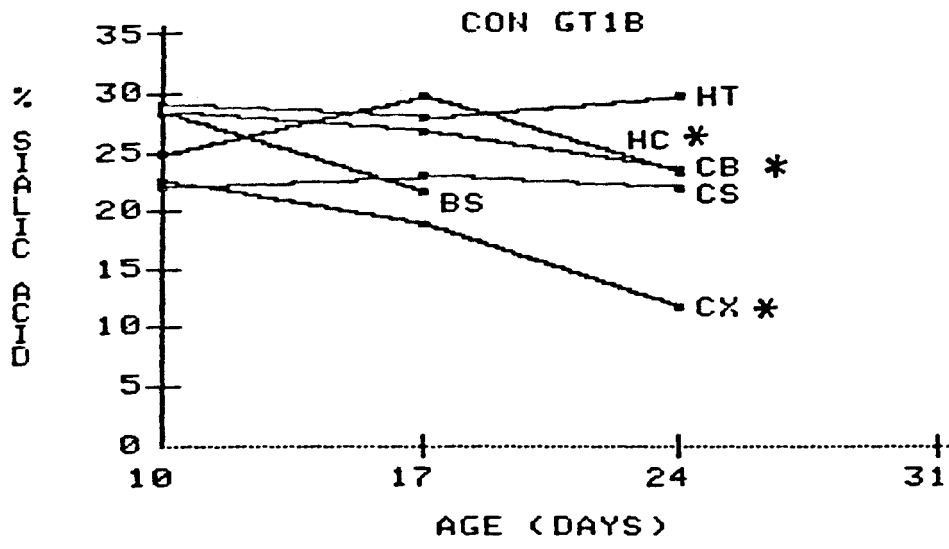


Figure 23: Developmental changes of GT<sub>1a</sub> in selected brain regions of control (C) rats.

The figure depicts percent distribution (% total ganglioside sialic acid) of ganglioside GT<sub>1a</sub> in the cerebral cortex (CX), cerebellum (CB), brain stem (BS), corpus striatum (CS), hypothalamus (HT) and hippocampus (HC) of control (C) (normal) rats with development. The percent distribution of GT<sub>1a</sub> was determined by densitometric scanning of GT<sub>1a</sub> on tlc plates.

Each point represents the mean value of six rats.

A \* indicates that there was a statistically significant ( $p < 0.05$ ) change in the percent distribution of the ganglioside during development in a particular brain region.

The standard deviations of each point are included in Tables A-I - A-VI of the Appendix.

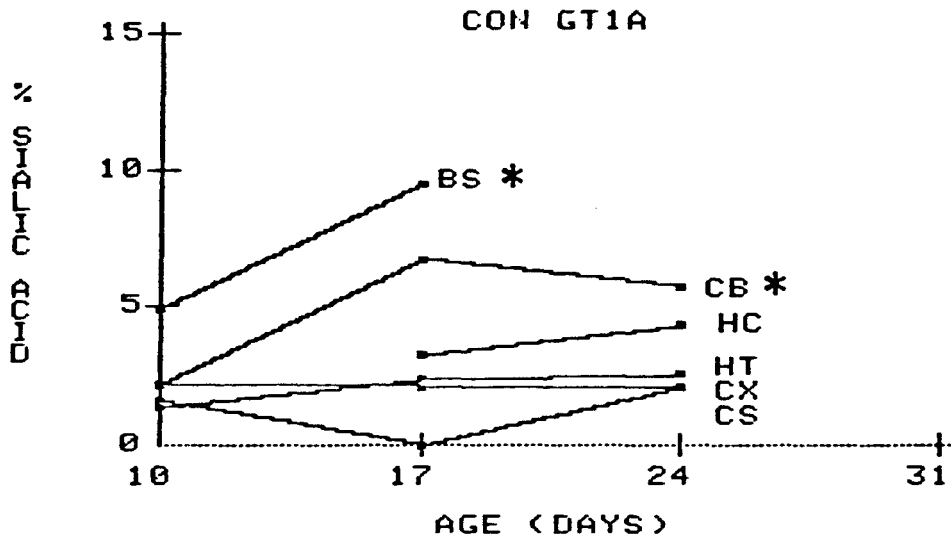


Figure 24: Developmental changes of  $GD_{1b}$  in selected brain regions of control (C) rats.

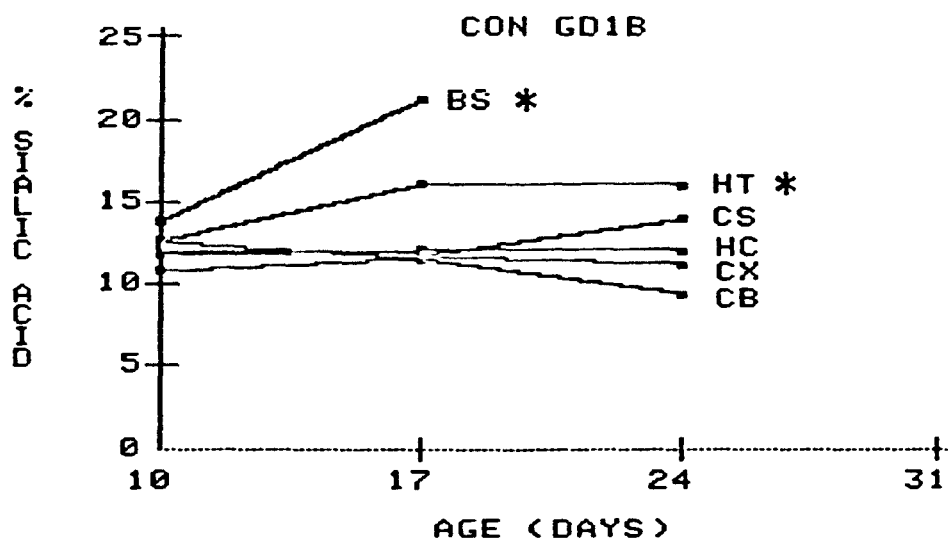
The figure depicts percent distribution (% total ganglioside sialic acid) of ganglioside  $GD_{1b}$  in the cerebral cortex (CX), cerebellum (CB), brain stem (BS), corpus striatum (CS), hypothalamus (HT) and hippocampus (HC) of control (C) (normal) rats with development. The percent distribution of  $GD_{1b}$  was determined by densitometric scanning of  $GD_{1b}$  on tlc plates.

Each point represents the mean value of six rats.

A \* indicates that there was a statistically significant ( $p < 0.05$ ) change in the percent distribution of the ganglioside during development in a particular brain region.

The standard deviations of each point are included in Tables A-I - A-VI of the Appendix.





GD<sub>1a</sub>

GD<sub>1a</sub> was the other predominant ganglioside species observed in most of the brain regions examined. The proportion of GD<sub>1a</sub> decreased significantly ( $p < 0.005$ ) during the 10 to 24 day age period in the hypothalamus (figure 25) but remained relatively constant in the other brain regions.

GD<sub>3</sub>

GD<sub>3</sub> was present in all the brain regions examined even though it was a minor constituent of the total ganglioside species. With development, the proportion of GD<sub>3</sub> showed an approximate two-fold increase ( $p < 0.005$ ) in the cerebral cortex and hypothalamus. Between 10 and 17 days the cerebellum also showed a significant increase ( $p < 0.005$ ) but the proportion returned to 10 day values by the 24th day (figure 26).

GM<sub>1</sub>

The most striking developmentally related increase in the proportion of GM<sub>1</sub> was observed in the cerebral cortex (figure 27). The proportion of GM<sub>1</sub> in the cerebral cortex increased two-fold and parallels the process of myelination. GM<sub>1</sub> is elevated in most white matter regions due to its predominance in myelin (Suzuki et al., 1967 and Ledeen et al., 1973). The hippocampus showed a significant

Figure 25: Developmental changes of  $GD_{1a}$  in selected brain regions of control (C) rats.

The figure depicts percent distribution (% total ganglioside sialic acid) of ganglioside  $GD_{1a}$  in the cerebral cortex (CX), cerebellum (CB), brain stem (BS), corpus striatum (CS), hypothalamus (HT) and hippocampus (HC) of control (C) (normal) rats with development. The percent distribution of  $GD_{1a}$  was determined by densitometric scanning of  $GD_{1a}$  on tlc plates.

Each point represents the mean value of six rats.

A \* indicates that there was a statistically significant ( $p < 0.05$ ) change in the percent distribution of the ganglioside during development in a particular brain region.

The standard deviations of each point are included in Tables A-I - A-VI of the Appendix.

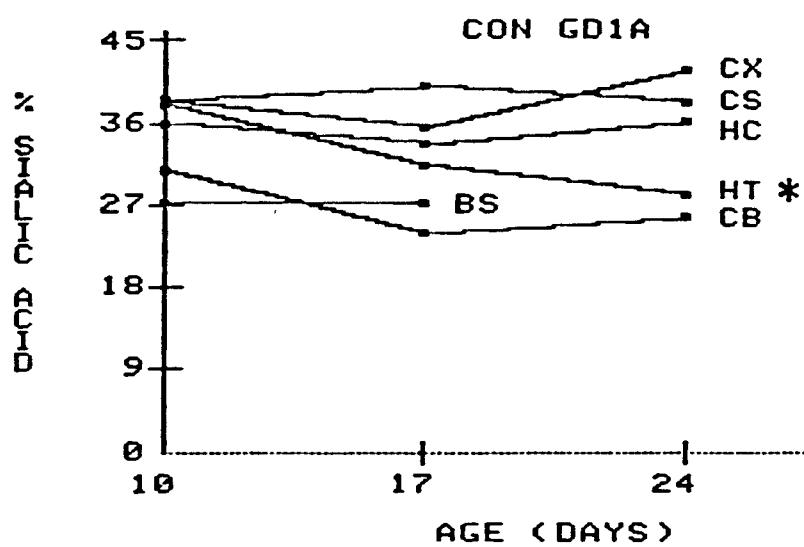


Figure 26: Developmental changes of  $GD_3$  in selected brain regions of control (C) rats.

The figure depicts percent distribution (% total ganglioside sialic acid) of ganglioside  $GD_3$  in the cerebral cortex (CX), cerebellum (CB), brain stem (BS), corpus striatum (CS), hypothalamus (HT) and hippocampus (HC) of control (C) (normal) rats with development. The percent distribution of  $GD_3$  was determined by densitometric scanning of  $GD_3$  on tlc plates.

Each point represents the mean value of six rats.

A \* indicates that there was a statistically significant ( $p < 0.05$ ) change in the percent distribution of the ganglioside during development in a particular brain region.

The standard deviations of each point are included in Tables A-I - A-VI of the Appendix.

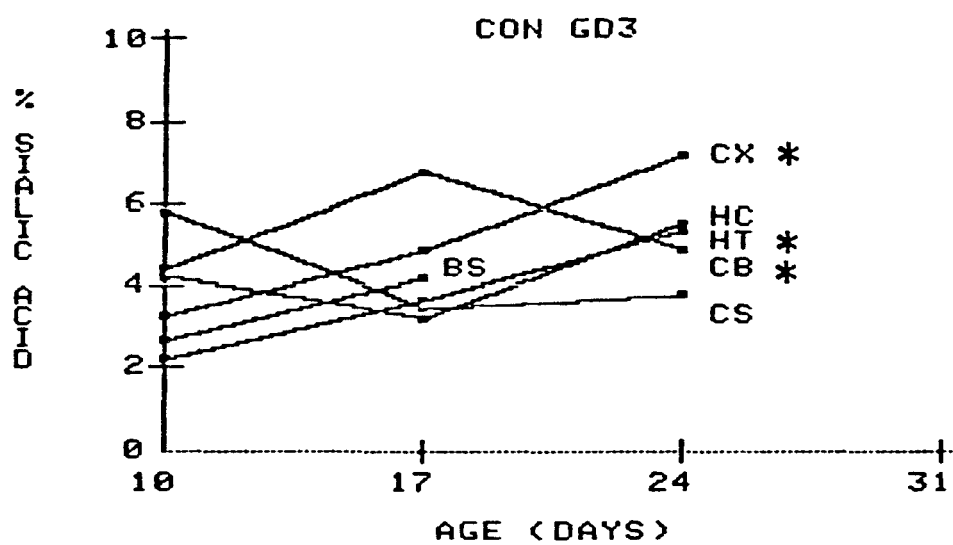


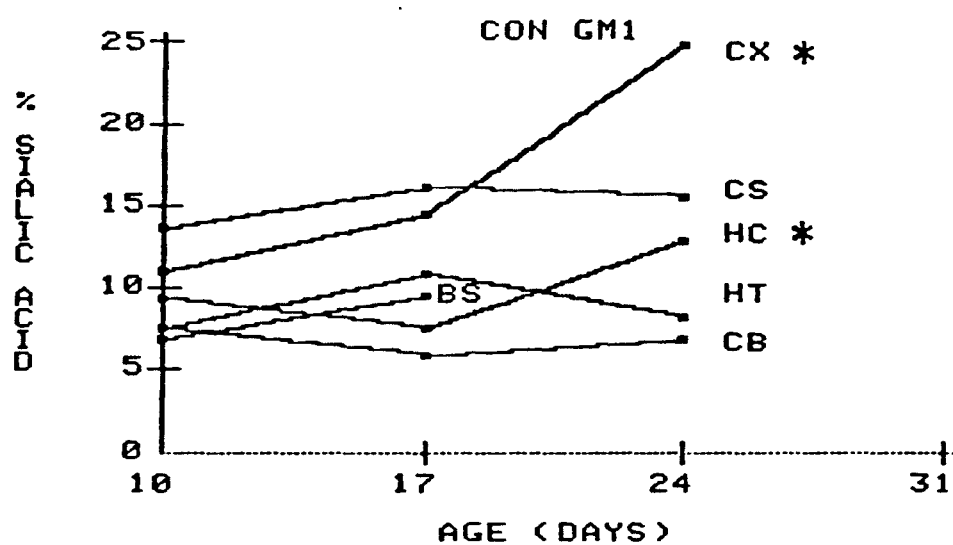
Figure 27 : Developmental changes of  $GM_1$  in selected brain regions of control (C) rats.

The figure depicts percent distribution (% total ganglioside sialic acid) of ganglioside  $GM_1$  in the cerebral cortex (CX), cerebellum (CB), brain stem (BS), corpus striatum (CS), hypothalamus (HT) and hippocampus (HC) of control (C) (normal) rats with development. The percent distribution of  $GM_1$  was determined by densitometric scanning of  $GM_1$  on tlc plates.

Each point represents the mean value of six rats.

A \* indicates that there was a statistically significant ( $p < 0.05$ ) change in the percent distribution of the ganglioside during development in a particular brain region.

The standard deviations of each point are included in Tables A-I - A-VI of the Appendix.





increase ( $p < 0.005$ ) in the proportion of  $GM_1$  between 17 and 24 days of age. In the remaining brain regions, the proportion of  $GM_1$  remained fairly constant throughout development.

#### Effect of Ethanol on the Percent Distribution of Individual Gangliosides in the Offspring

Statistically significant differences ( $p < 0.05$ ) between ethanol and control offspring were observed in the percent distribution of individual gangliosides in most of the brain regions examined (figures 28-33).

The hippocampus, hypothalamus and the corpus striatum were the regions that were most affected. The hippocampus (figure 28) showed statistically significant increases in the ethanol pups in the proportions of  $GT_{1b}$  at 10 days,  $GD_{1b}$  at 17 days, as well as  $GQ_{1b}$  and  $GT_{1a}$  at 24 days. Decreases were observed in  $GM_1$  at 10 days and in  $GD_{1b}$  at 24 days of age.

The hypothalamus (figure 29) showed the greatest number of statistically significant differences at the 24 day age period. Ethanol pups had an increase in  $GT_{1a}$  and  $GD_{1b}$  and a decrease in  $GD_3$ . Significant differences were also observed in the proportions of  $GD_{1a}$  and  $GM_1$  at the 17 day age period.

In the corpus striatum (figure 30), significant differences were observed in the proportion of most of

Figure 28: Gangliosides of the Hippocampus.

The figure depicts the percent distribution of sialic acid among individual ganglioside of the hippocampus of control (C) and ethanol (E) rats at 10, 17, and 24 days of age. Gangliosides from each control (C) and ethanol (E) rat were separated by tlc. The percent distribution of individual gangliosides was directly determined by densitometric scanning of the resorcinol positive spots as described by Ando et al. (1978).

Each bar represents the mean value of six rats.

A \* indicates that the comparison of values from control (C) and ethanol (E) rats are statistically significant ( $p < 0.05$ ) using the student's 't' test.

The standard deviations of each bar are included in Table A-IV in the Appendix.

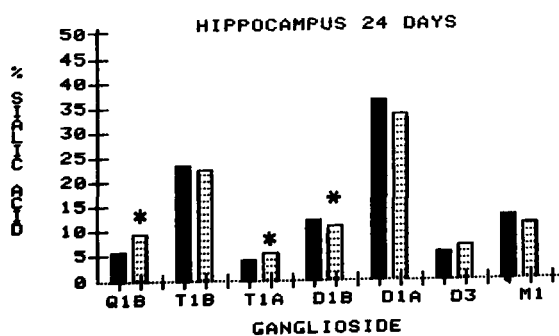
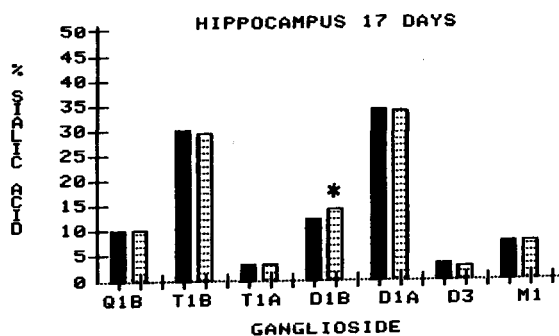
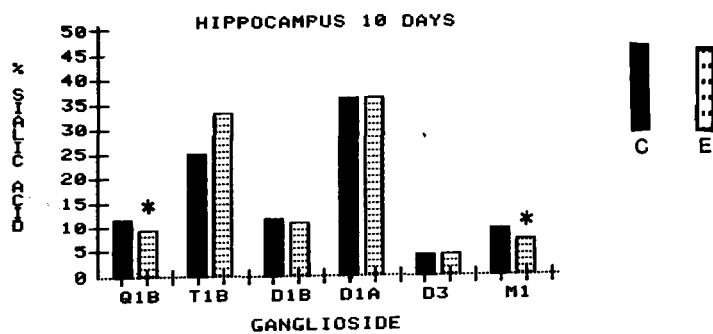


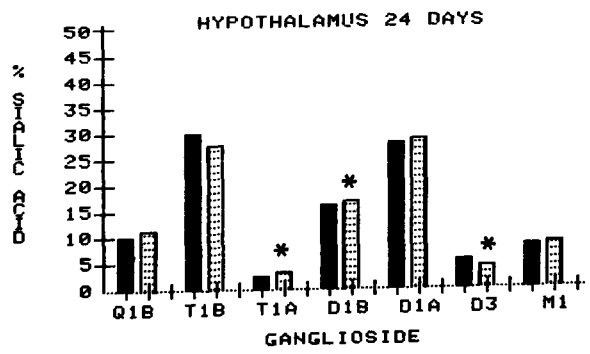
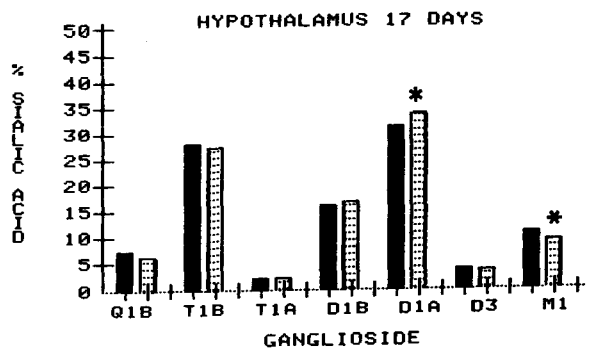
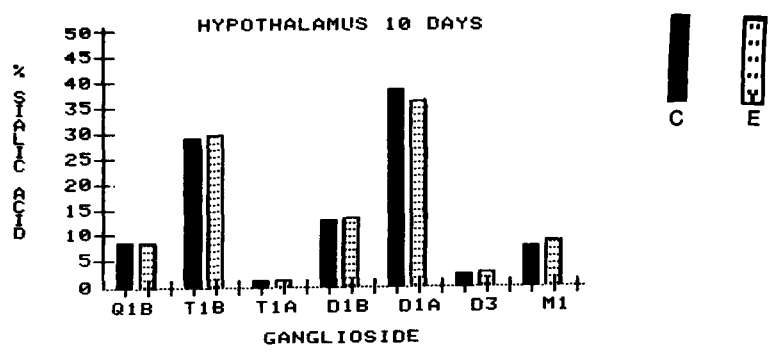
Figure 29: Gangliosides of the Hypothalamus.

The figure depicts the percent distribution of sialic acid among individual ganglioside of the hypothalamus of control (C) and ethanol (E) rats at 10, 17, and 24 days of age. Gangliosides from each control (C) and ethanol (E) rat were separated by tlc. The percent distribution of individual gangliosides was directly determined by densitometric scanning of the resorcinol positive spots as described by Ando et al. (1978).

Each bar represents the mean value of six rats.

A \* indicates that the comparison of values from control (C) and ethanol (E) rats are statistically significant ( $p < 0.05$ ) using the student's 't' test.

The standard deviation of each bar are included in Table A-VI in the Appendix.



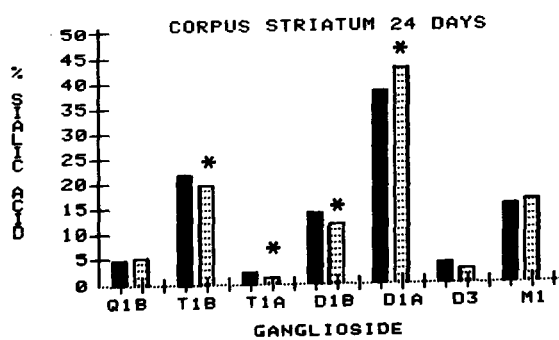
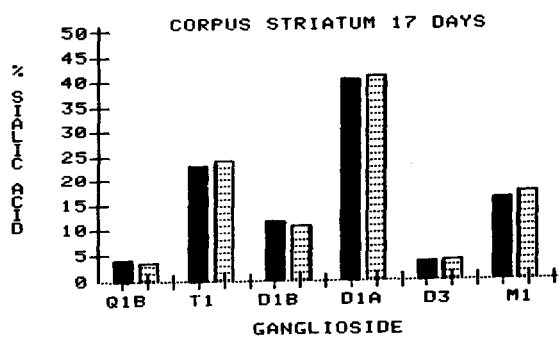
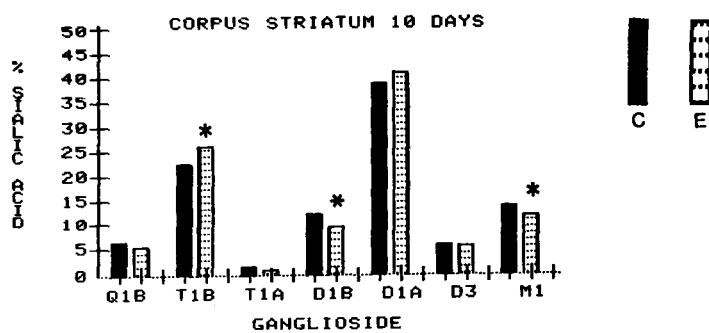
## Figure 30: Gangliosides of the Corpus Striatum.

The figure depicts the percent distribution of sialic acid among individual ganglioside of the corpus striatum of control (C) and ethanol (E) rats at 10, 17, and 24 days of age. Gangliosides from each control (C) and ethanol (E) rat were separated by tlc. The percent distribution of individual gangliosides was directly determined by densitometric scanning of the resorcinol positive spots as described by Ando et al. (1978).

Each bar represents the mean value of six rats.

A \* indicates that the comparison of values from control (C) and ethanol (E) rats are statistically significant ( $p < 0.05$ ) using the student's 't' test.

The standard deviations of each bar are included in Table A-V in the Appendix.



the individual gangliosides at 24 days. Increases were observed in  $GD_{1a}$  while the trisialogangliosides  $GT_{1b}$  and  $GT_{1a}$  as well as  $GD_{1b}$  showed significant decreases. In addition, significant differences between ethanol and control pups were also seen in  $GT_{1b}$ ,  $GD_{1b}$  and  $GM_1$  at 10 days of age.

The cerebral cortex (figure 31) showed comparatively few significant differences at the early age periods and by 24 days no statistically significant differences were observed between control and ethanol pups.

In the brain stem (figure 32) and the cerebellum (figure 33) the ethanol and control pups had a similar pattern in the distribution of sialic acid among the individual gangliosides at all the ages examined. The only exception was a statistically significant increase in the proportion of the polysialoganglioside  $GQ_{1b}$  in the ethanol pups at 10 days of age.

### Radiolabelled Brain Regional Gangliosides

The percent distribution of individual radiolabelled gangliosides associated with different brain regions at 10 , 17 and 24 days of age are included in Tables A-VII- A-XII in the Appendix; comparisons between ethanol and matched-control pups are also included in these tables.

Studies with radiolabelled precursors reflect the synthesis or the incorporation of the radiolabel into newly



Figure 31: Gangliosides of the Cerebral Cortex.

The figure depicts the percent distribution of sialic acid among individual ganglioside of the cerebral cortex of control (C) and ethanol (E) rats at 10, 17, and 24 days of age. Gangliosides from each control (C) and ethanol (E) rat were separated by tlc. The percent distribution of individual gangliosides was directly determined by densitometric scanning of the resorcinol positive spots as described by Ando et al. (1978).

Each bar represents the mean value of six rats.

A \* indicates that the comparison of values from control (C) and ethanol (E) rats are statistically significant ( $p < 0.05$ ) using the student's 't' test.

The standard deviations of each bar are included in Table A-I in the Appendix.

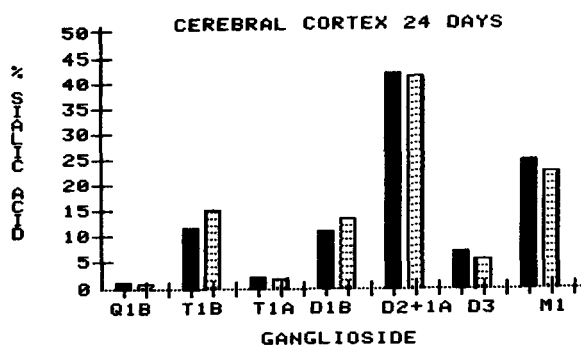
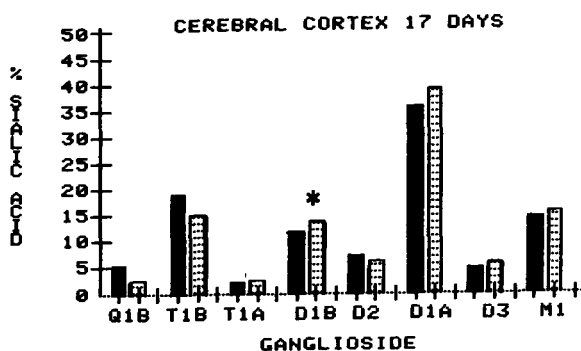
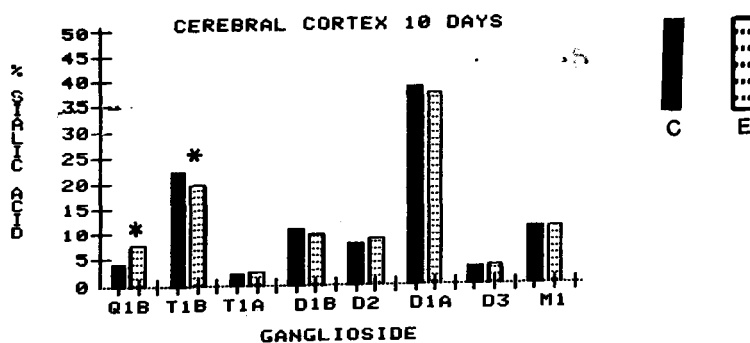


Figure 32: Gangliosides of the Brain Stem.

The figure depicts the percent distribution of sialic acid among individual ganglioside of the brain stem of control (C) and ethanol (E) rats at 10, 17, and 24 days of age. Gangliosides from each control (C) and ethanol (E) rat were separated by tlc. The percent distribution of individual gangliosides was directly determined by densitometric scanning of the resorcinol positive spots as described by Ando et al. (1978).

Each bar represents the mean value of six rats.

A \* indicates that the comparison of values from control (C) and ethanol (E) rats are statistically significant ( $p < 0.05$ ) using the student's 't' test.

The standard deviations of each bar are included in Table A-III in the Appendix.

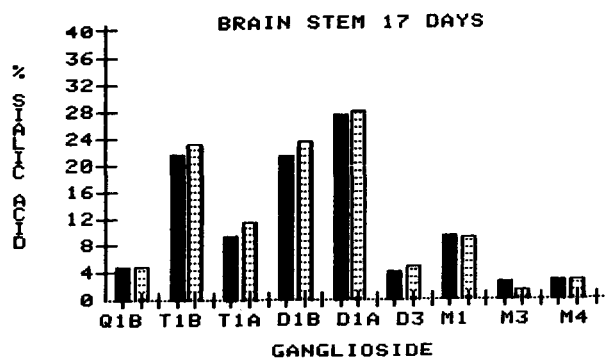
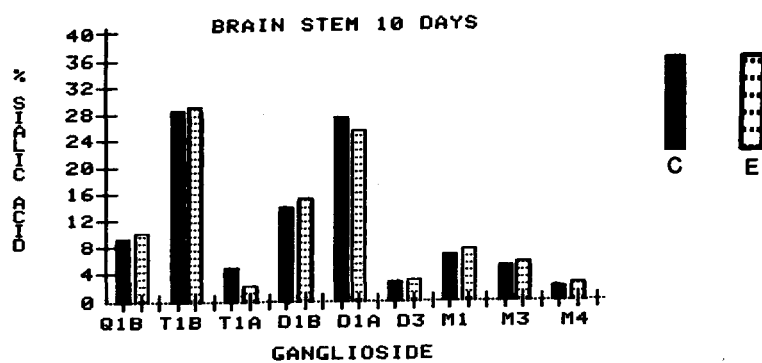


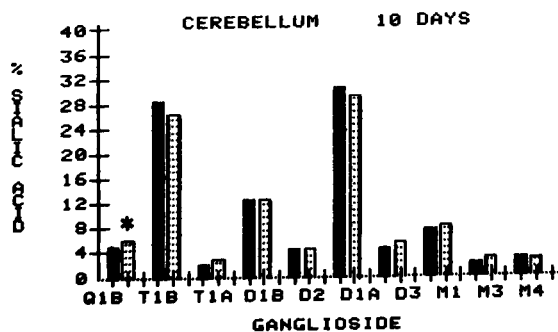
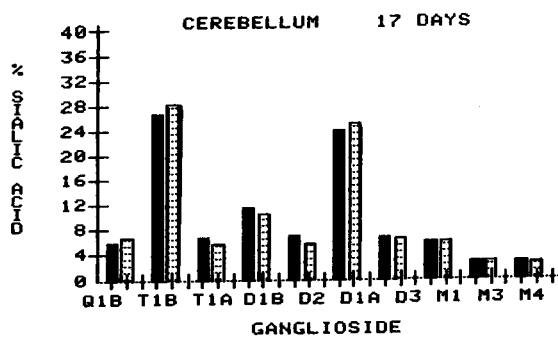
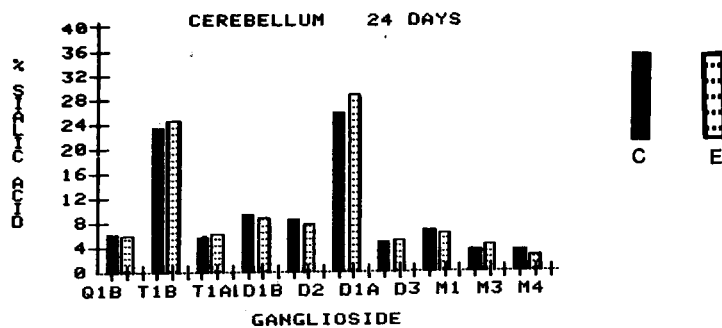
Figure 33: Gangliosides of the Cerebellum.

The figure depicts the percent distribution of sialic acid among individual ganglioside of the cerebellum of control (C) and ethanol (E) rats at 10, 17, and 24 days of age. Gangliosides from each control (C) and ethanol (E) rat were separated by tlc. The percent distribution of individual gangliosides was directly determined by densitometric scanning of the resorcinol positive spots as described by Ando et al. (1978).

Each bar represents the mean value of six rats.

A \* indicates that the comparison of values from control (C) and ethanol (E) rats are statistically significant ( $p < 0.05$ ) using the student's 't' test.

The standard deviations of each bar are included in Table A-II in the Appendix.



synthesized gangliosides. On the other hand, studies of relative distribution of sialic acid among gangliosides reflects the distribution of gangliosides which have previously been synthesized. As seen in Tables A-VII - A-XII in the Appendix, a major proportion of the label was incorporated into the disialoganglioside  $GD_{1a}$  in all the brain regions examined. Radioactivity was also detected in the other gangliosides.

Statistically significant differences ( $p < 0.05$ ) were observed between ethanol and control offspring in the percent distribution of individual radiolabelled gangliosides in most of the brain regions examined.

In the cerebral cortex (figure 34), transient abnormalities were observed in several of the gangliosides. At 17 days of age, a decrease was observed in the disialogangliosides  $GD_{1b}$ ,  $GD_2$  and  $GD_3$  and an increase was found in  $GM_1$ . Furthermore, persistent decreases were observed in the synthesis of  $GD_3$ . Abnormalities were also observed in  $GM_1$  at 17 and 24 days.

In the cerebellum (figure 35) a higher proportion of radioactivity was consistently found associated with the trisialogangliosides ( $GT_{1b}$  and  $GT_{1a}$ ) in the ethanol pups. This difference was statistically significant ( $p < 0.05$ ) at 24 days of age. Abnormalities were also observed with the radioactivity associated with  $GM_1$ .

The brain stem (figure 36) showed similar patterns

Figure 34: Radiolabeled Gangliosides of the Cerebral Cortex.

The figure depicts the relative distribution (% total dpm) of radioactivity of individual ganglioside sialic acid solubilized from tlc.lanes, loaded with mixtures of gangliosides from the cerebral cortex of a control (C) rat and an ethanol (E) rat at 10, 17, and 24 days of age.

Each bar represents the mean value of six rats.

A \* indicates that the difference of values from control (C) and ethanol (E) rats is statistically significant ( $p < 0.05$ ) using the student's 't' test.

The standard deviations of each bar are included in Table A-VII in the Appendix.



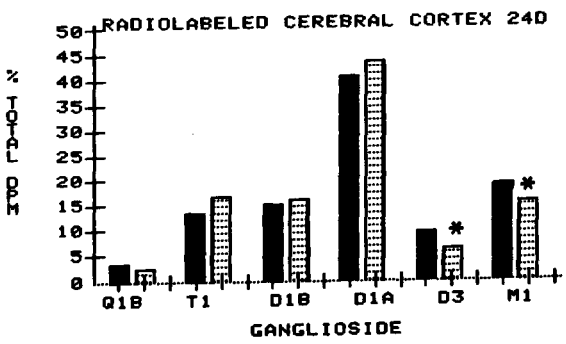
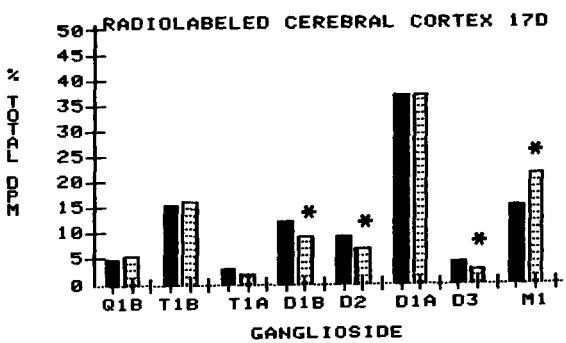
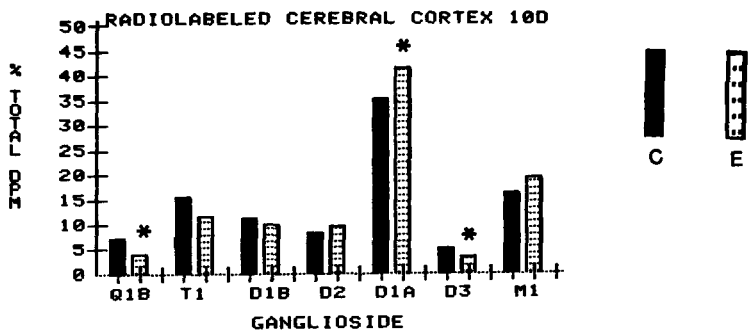


Figure 35: Radiolabeled Gangliosides of the Cerebellum.

The figure depicts the relative distribution (% total dpm) of radioactivity of individual ganglioside sialic acid solubilized from tlc lanes, loaded with mixtures of gangliosides from the cerebellum of a control (C) rat and an ethanol (E) rat at 10, 17, and 24 days of age.

Each bar represents the mean value of six rats.

A \* indicates that the difference of values from control (C) and ethanol (E) rats is statistically significant ( $p < 0.05$ ) using the student's 't' test.

The standard deviations of each bar are included in Table A-VIII in the Appendix.

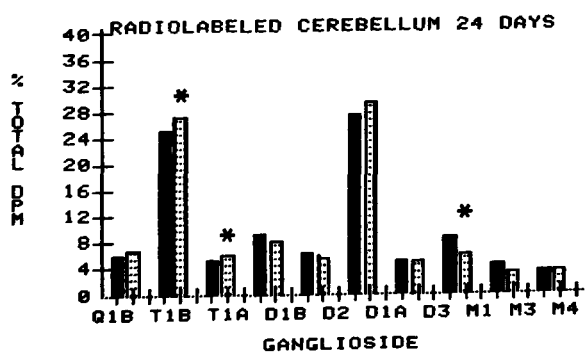
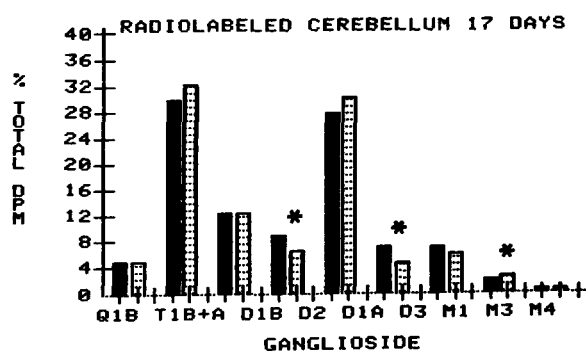
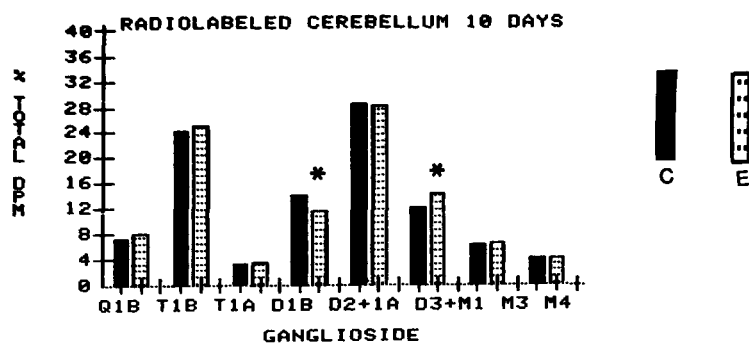


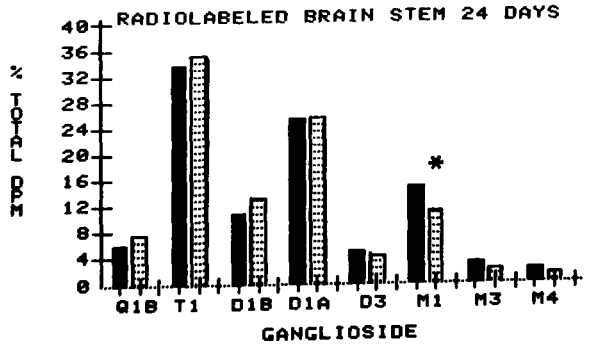
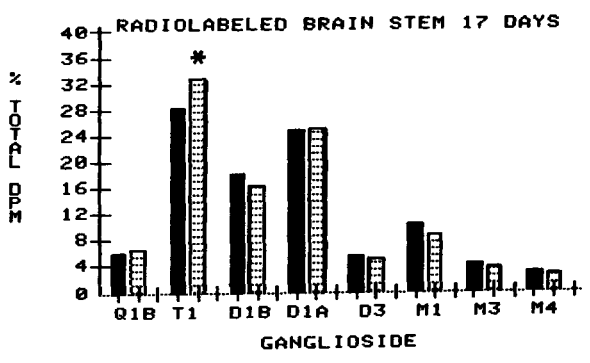
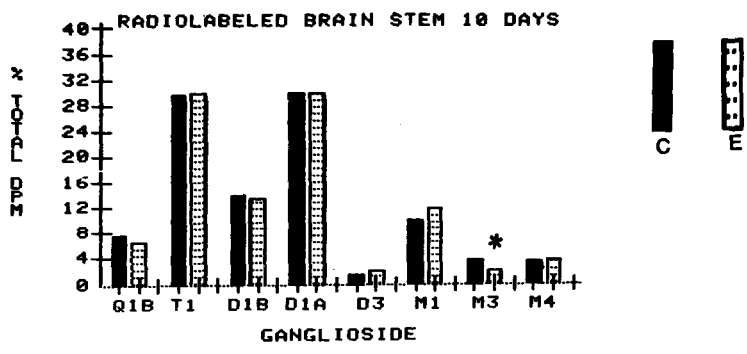
Figure 36: Radiolabeled Gangliosides of the Brain Stem.

The figure depicts the relative distribution (% total dpm) of radioactivity of individual ganglioside sialic acid solubilized from tlc lanes, loaded with mixtures of gangliosides from the brain stem of a control (C) rat and an ethanol (E) rat at 10, 17, and 24 days of age.

Each bar represents the mean value of six rats.

A \* indicates that the difference of values from control (C) and ethanol (E) rats is statistically significant ( $p < 0.05$ ) using the student's 't' test.

The standard deviations of each bar are included in Table A-IX in the Appendix.



in the relative incorporation of radioactivity into most of the gangliosides. However, a statistically significant decrease ( $p < 0.05$ ) in the relative incorporation of isotope into  $GM_1$  was found at 24 days.

The hippocampus (figure 37) showed many transient abnormalities. A significantly higher incorporation in the radioactivity ( $p < 0.05$ ) associated with  $GD_{1a}$  and a decrease in  $GT_{1a}$  and  $GD_{1b}$  was observed at 10 days of age. Differences were also observed with  $GM_1$  at 17 days and  $GD_3$  at 24 days of age.

In the corpus striatum (figure 38) differences were observed in the radioactivity associated with the trisialo-gangliosides. In the ethanol pups, these gangliosides consistently had a higher proportion of radioactivity associated with them which was statistically significant ( $p < 0.05$ ) at 10 and 17 days of age. Persistent abnormalities were also observed with  $GD_3$ . The ethanol pups showed significant decreases in the radioactivity associated with  $GD_3$  at 17 as well as 24 days of age.

The hypothalamus (figure 39) had similar patterns in the relative incorporation of radioactivity associated with the more complex gangliosides in control and ethanol pups at 24 days of age. However, significant decreases ( $p < 0.05$ ) in the ethanol pups were observed with the radioactivity associated with  $GD_3$  and  $GM_1$ .

Figure 37: Radiolabeled Gangliosides of the Hippocampus.

The figure depicts the relative distribution (% total dpm) of radioactivity of individual ganglioside sialic acid solubilized from tlc lanes, loaded with mixtures of gangliosides from the hippocampus of a control (C) rat and an ethanol (E) rat at 10, 17, and 24 days of age.

Each bar represents the mean value of six rats.

A \* indicates that the difference of values from control (C) and ethanol (E) rats is statistically significant ( $p < 0.05$ ) using the student's 't' test.

The standard deviations of each bar are included in Table A-X in the Appendix.

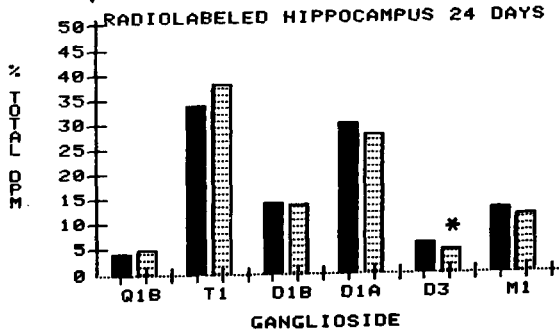
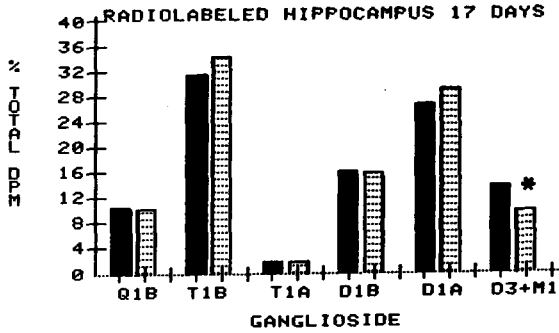
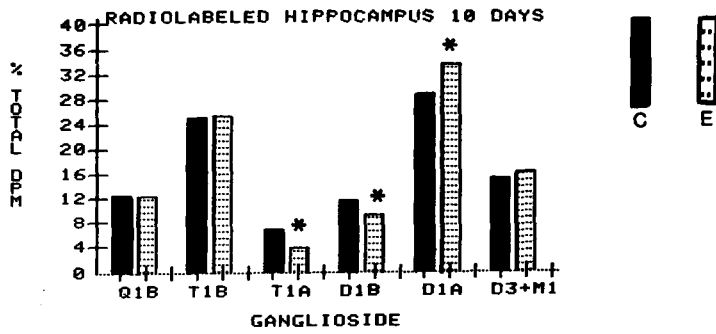




Figure 38: Radiolabeled Gangliosides of the Corpus Striata.

The figure depicts the relative distribution (% total dpm) of radioactivity of individual ganglioside sialic acid solubilized from tlc lanes, loaded with mixtures of gangliosides from the corpus striata of a control (C) rat and an ethanol (E) rat at 10, 17, and 24 days of age.

Each bar represents the mean value of six rats.

A \* indicates that the difference of values from control (C) and ethanol (E) rats is statistically significant ( $p < 0.05$ ) using the student's 't' test.

The standard deviations of each bar are included in Table A-XI in the appendix.

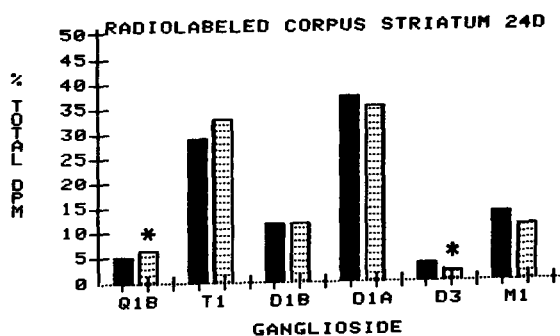
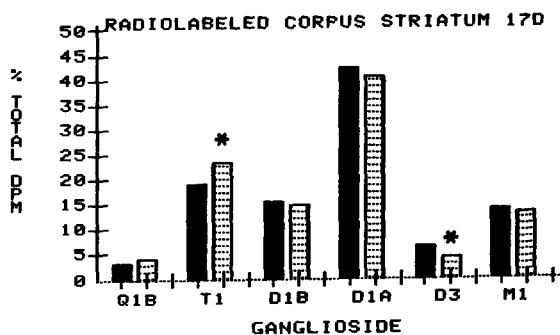
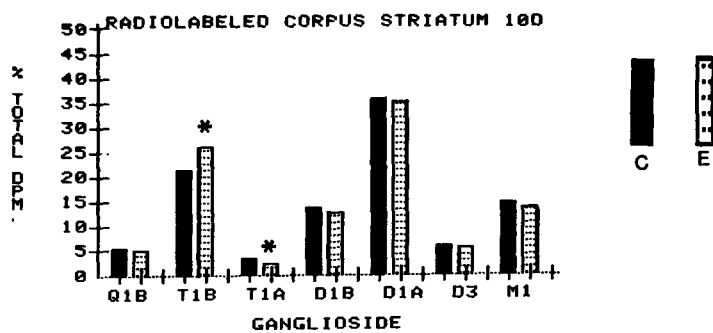


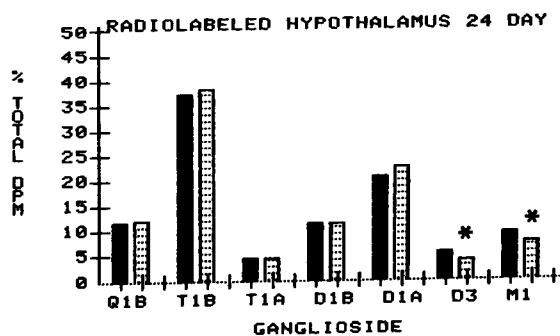
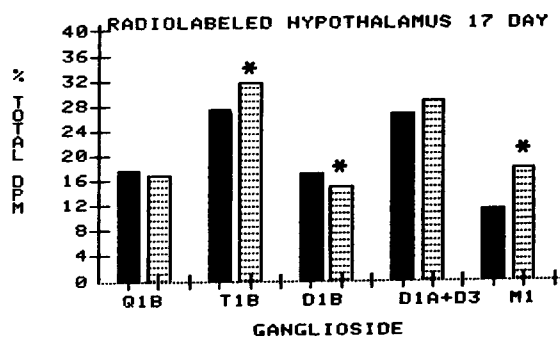
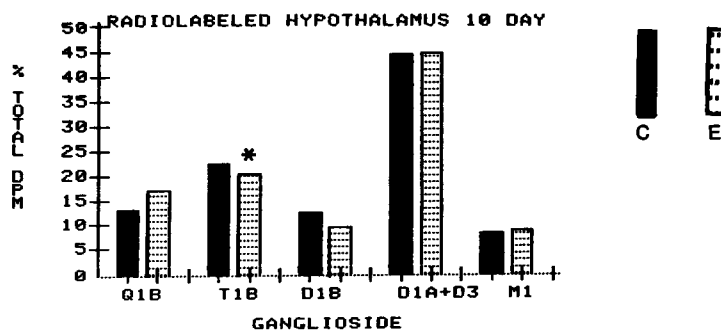
Figure 39: Radiolabeled Gangliosides of the Hypothalamus.

The figure depicts the relative distribution (% total dpm) of radioactivity of individual ganglioside sialic acid solubilized from tlc anes, loaded with mixtures of gangliosides from the hypothalamus of a control (C) rat and an ethanol (E) rat at 10, 17, and 24 days of age.

Each bar represents the mean value of six rats.

A \* indicates that the difference of values from control (C) and ethanol (E) rats is statistically significant ( $p < 0.05$ ) using the student's 't' test.

The standard deviations of each bar are included in Table A-XII in the Appendix.



### Sialic Acid Concentration of Brain Regions

The ganglioside sialic acid concentration of the six brain regions were examined at three different age periods (Table A-XIII of the Appendix). Over the 10 to 24 day age range examined, the sialic acid concentration of the cerebellum, hypothalamus and hippocampus increased approximately two-fold. In these brain regions, the rise in concentration of ganglioside sialic acid was moderate between 10 and 17 days. However, a dramatic increase was observed between 17 and 24 days of age. The other brain regions showed relatively similar concentrations of ganglioside sialic acid at all ages. Comparable concentrations of ganglioside sialic acid were observed in both ethanol and matched-control pups at all the age periods examined.

### Summary of Brain Regional Gangliosides

From the data presented, it is evident that the concentration of ganglioside sialic acid, the percentage of individual gangliosides as well as the proportion of radioactivity associated with individual gangliosides differs from one region to another. In most of the brain regions examined,  $GD_{1a}$  appeared to be the predominant ganglioside. However, in the cerebellum and brain stem, both  $GT_{1b}$  and  $GD_{1a}$  were present in relatively similar proportions. The most striking observation was the decrease in the proportion of  $GD_{1a}$  in the hypothalamus with development, thus resulting

in relatively similar proportions of  $GD_{1a}$  and  $GT_{1b}$  by about the 24-day age period (Table A-V of the Appendix).

The results presented also demonstrate that maternal ethanol consumption results in abnormalities both in the synthesis as well as the proportion of individual gangliosides. The particular gangliosides that are affected vary with age and brain region.

## CHAPTER IV

### DISCUSSION

In our studies, the chronic rat model was used to parallel the chronic ethanol intake of many alcoholic women. Accordingly, ethanol was administered for 6-8 weeks prior to mating and throughout gestation. The mothers were withdrawn from the alcohol after they had given birth because alcohol may decrease milk output. Alcohol has been shown to inhibit oxytocin which is necessary for lactation (Fuchs and Wagner, 1963).

The incidence of conception, duration of gestation, litter size and infant or neonatal mortality in our ethanol-treated rats were comparable to those seen in pair-fed controls. This is in contrast to the observation of Tze and Lee (1975), Pilstrom and Kiessling (1976), Kornick (1976) and Henderson and Schenker (1977). Differences in results may be due to differences in the timing, duration and amount of alcohol used in these studies or to nutritional effects. It should be noted that our conclusions are based on a large number of litters (40-45). Our studies also clearly demonstrate that chronic maternal alcoholism does not affect the brain weights and the brain protein content of the offspring that are at least 10 days of age. This laboratory routinely finds normal or

near-normal brain and body weights for ethanol pups at the ages (>10days) we examined (Druse and Hofteig, 1977; Hofteig and Druse, 1978; Druse et al., 1981). It is possible that the brain weights are affected at younger ages (closer to the time of the last exposure to ethanol). Others have reported that postnatal administration of ethanol until the age of sacrifice results in significantly reduced brain weights (Diaz and Samson, 1980).

In addition, the yield of synaptic plasma membranes from the 10 to 24 day-old offspring of ethanol and pair-fed control pups were found to be comparable. This finding differs from observed SPM deficit in undernourished animals (Smith and Druse, in press) and suggests that the control and ethanol pups were comparably nourished. The yield of SPMs from the offspring of rats fed the revised diet (\*C-\*C and \*E-\*E) were roughly comparable to those of the offspring of chow-fed rats (Smith and Druse, in press). However, the yield of SPMs from the offspring of rats fed both of the original diets (C-C and E-E) was considerably less than that of similar offspring of rats that were fed the revised diets (\*C-\*C and \*E-\*E). Despite the normal brain weights and brain protein content in the C-C and E-E pups, it is possible that both these animals were undernourished in utero relative to rats in the other groups. Normal brain weights and decreased myelin protein have been found in 17 and 54 day-old gestationally undernourished rats (Figlewicz, 1979).



Our findings support a previous study (Weiner et al., 1981) that emphasized the importance of using nutritionally adequate liquid diets in 'fetal alcohol' studies.

It should be noted that the present study is a comparative study of the yield of SPMs obtained by identical methods from control and ethanol pups. Comparisons of the quantity of SPM proteins isolated from normal developing rats have been made (Kelly and Cotman, 1981). The absolute yield of SPMs and synaptosomes have been shown to be influenced by differences in the extent of tissue homogenization (Cotman, 1974), the source (whole brain or selected brain region, animal, age etc.) of synaptic plasma membrane and the method of removing supernatants from synaptosomal and SPM pellets (Smith and Druse, in press).

Despite the comparable yields of SPMs from ethanol and matched control pups, the ethanol pups demonstrated SPM glycoprotein abnormalities. These abnormalities were most severe in the non-cross-fostered rats whose mothers consumed the original diet although abnormalities were also found in the cross-fostered offspring of rats that received the revised liquid diets. There are several explanations for the altered patterns of fucosylation of SPM glycoproteins

1. Altered synthesis of individual polypeptide 'backbones' of glycoproteins would provide altered levels of precursors for the addition of the carbohydrate moieties.
2. Since fucose is a terminal sugar,

normal fucosylation depends on the presence of a nearly-complete (and normal) glycoprotein structure. Thus, altered activity of any of the enzymes or substrates involved in the addition of the carbohydrate constituents to the polypeptide 'backbones' could result in an abnormal synthesis of fucosylated SPM glycoproteins. This possibility has not been examined in this dissertation and further studies of this possibility are warranted. Nonetheless, it should be noted that the effects observed in the present study are not directly due to the depressant effect of ethanol or its metabolite (acetaldehyde) on protein synthesis (Tewari and Noble, 1971; Noble and Tewari, 1973; Sorrell and Tuma, 1978 and Tuma and Sorrell, 1981). Whereas the presence of ethanol (or acetaldehyde) in vitro or in vivo reportedly has the mentioned effects, no information is available about protein or glycoprotein synthesis one or more weeks after the last exposure to ethanol. 3. The observed persistent abnormalities could be due to an abnormal development and/or maturation of the brain (i.e. brain synapses and SPM glycoprotein patterns)(Fu et al., 1981). Prenatal ethanol exposure has been shown to affect hippocampal mossy fibers (West et al., 1981) and pyramidal cells (Barnes and Walker, 1981; Sherwin et al., 1981) and neocortical pyramidal cells (Hammer and Scheibel, 1981). 4. Alternatively, it is also possible that administration of ethanol during the entire prenatal period caused selective nutritional deficiencies

by inhibiting the placental transport of essential nutrients (Henderson et al., 1981). Severe postnatal and prenatal undernutrition are known to result in differences in the synthesis of fucosylated SPM glycoproteins (Smith and Druse, 1980; 1981; Druse et al., 1982; Tonetti and Druse, unpublished observations). However, our results indicate that chronic maternal alcoholism has a greater effect on fucosylation of SPM proteins and results in abnormalities in the synthesis of a larger number of fucosylated SPM glycoproteins than in prenatal or postnatal undernutrition.

One must also consider the possibility that the purity of SPMs from ethanol and control pups differed in a way that could result in altered glycoprotein patterns. However, an electron micrograph study of synaptosomes isolated from both control and ethanol exposed offspring failed to show any differences in the morphology of the synaptosomes isolated from either offspring (Project undertaken by graduate student Doris Mangiaracina as part of the requirement for the Electron Microscopy course). Other potential explanations for the differences between control and ethanol pups (e.g., altered bouyancy, fragility of SPMs) cannot be excluded. However, in light of the comparable yield of SPMs from control and age- and diet-matched ethanol pups, this hypothesis seems unlikely.

The observed SPM glycoprotein abnormalities in E-E and \*E-Ch rats differ from the normal pattern of synthesis

of SPM glycoproteins found in the offspring of rats that were pair-fed the \*C and \*E diets only during the last trimester of gestation (Druse et al., 1981). Undoubtedly, the metabolic and hormonal abnormalities that accompany chronic alcoholism (Isselbacher, 1977), the increased length of in utero ethanol (and metabolite exposure) in the chronic study, and the higher dose of ethanol consumed by the tolerant chronic group (Druse et al., 1981) contributed to the observed differences.

It should be noted that the pattern of fucose radioactivity incorporated in SPM glycoproteins in the non-cross-fostered control animals (C-C) was different from that of the other control animals (see Appendix, Figures A-1 to A-9). This may indicate that the original liquid diet was nutritionally inadequate. In support of this idea is the lower yield of SPMs from control offspring whose mothers consumed the original diet (C-C) than that from the offspring of mothers that were fed the revised liquid diets (Table VIII). In addition, nutritional stress has previously been shown to affect both the yield of SPMs and the synthesis of SPM glycoproteins (Druse et al., 1982; Smith and Druse, in press). However, since the present study was a comparative study, differences between the ethanol and matched control pups would be expected to be due primarily to the effects of ethanol exposure.

In order to avoid potential nutritional and

maturational problems many laboratories routinely cross-foster the offspring of rats that were fed ethanol or control liquid diets to normal chow-fed mothers (Abel and Dintcheff, 1978; Detering et al., 1979). Cross-fostering also eliminates problems of maintaining ethanol offspring with mothers who may have alcohol- or withdrawal-related hormonal and metabolic disturbances. Certainly the high (though not significantly higher) SPM yield from cross-fostered pups support the use of the cross-fostered protocol. The use of the reverse cross-fostered group also proved important. The results obtained from this group clearly demonstrated the SPM glycoprotein abnormalities were only present in the biologic offspring of 'alcoholic rats'. This observation was particularly interesting as most of the synaptogenesis in rat brain occurs post-natally during the suckling period (Jacobson, 1978). Thus the inclusion of the reverse cross-fostered rats also allowed us to conclude that the glycoprotein abnormalities are primarily due to a prenatal effect and are not exclusively dependent on the status (i.e. hormonal, metabolic, etc.) of the lactating dams. Although the cross-fostering and reverse cross-fostering protocols are important, one wonders, however, if the diet of an alcoholic woman is not more like that of the original (slightly malnourished ethanol diet.

In summary, it appears that chronic maternal ethanol consumption has a severe effect on the synthesis of SPM glycoproteins in developing offspring, without affecting the content of SPMs per se. This abnormality seems to be augmented by poor nutrition though the nutritional status is not solely responsible for these effects. At the present, the functional significance of the observed SPM glycoprotein abnormalities is not understood. However, in view of the postulated important roles of glycoproteins in cell adhesion and synaptic connectivity (Brunngraber, 1969; Barondes, 1970; Mahler, 1979) and as components of neurotransmitter receptors (Brunngraber, 1979; Salvatera and Matthews, 1980), any alteration in their synthesis during the studied period of active synaptogenesis could seriously affect normal synaptic development and function.

#### Whole Brain Microsomal, Synaptosomal and Synaptic Plasma Membrane Gangliosides

The concentration of ganglioside sialic acid in the microsomal membranes was 20.6  $\mu\text{g}$  of sialic acid per mg of protein at 24 days. Our value for the 24 day age is higher than that reported by Avrova et al. (1973), Tettamanti et al. (1973), Tamai et al. (1974) and Skrivanek

et al. (1981) for a similar age period. However, our results are similar to that of Yohe et al. (1980) who reported a microsomal ganglioside concentration of 22.4  $\mu\text{g}$ . In agreement with the observations of Yohe et al. (1980), our results also indicated that the concentration of gangliosides in the synaptosomal membrane was approximately half of that in the microsomal membranes. The concentration of ganglioside sialic acid in the synaptosomal membranes in our study is comparable to that of other investigators (Seminario et al., 1964; Dekirmenjian and Brunngraber, 1969; Avrova et al., 1973; Caputto et al., 1974 and Yohe et al., 1980).

The percent distribution of radioactivity associated with the newly synthesized gangliosides of the microsomal and synaptosomal fractions was quite similar. Such similarities were also reported by Yohe et al., (1980). Our results indicate that approximately 3-5% of the total radioactivity in the microsomal membranes was associated with the monosialogangliosides  $\text{GM}_2$ ,  $\text{GM}_3$  and  $\text{GM}_4$ . These gangliosides appear to be localized predominantly in myelin membranes (Ledeen et al., 1973). Thus, the negligible or trace amounts of radioactivity associated with these gangliosides in the synaptosomal membranes suggest very little contamination of these membranes with myelin membrane fractions.

The concentration of ganglioside sialic acid in synaptic plasma membranes was found to be 24.7  $\mu\text{g}$  sialic

acid per mg of protein at 24-days in the present study. Values reported in the literature for SPM have been inconsistent. There is a six-fold difference between the highest (40-45  $\mu\text{g}$  sialic acid/mg protein, Breckenridge et al., 1972 and Avrova et al., 1973) and the lowest values (7.3  $\mu\text{g}$  sialic acid/mg protein, Lapetina et al., 1968). However, most of the reported values are in the 16-21  $\mu\text{g}$  range (Brunngraber et al., 1967; Avrova et al., 1973; Rappart and Mahadik, 1978; Skrivanek et al., 1978 and Hungund and Mahadik, 1981).

The percent distribution of sialic acid among individual SPM gangliosides was obtained from densitometric scans of chromatographed plates. In agreement with other studies (Breckenridge et al., 1972; Avrova et al., 1973 and Cruz and Gurd, 1981), our results indicate that the major gangliosides associated with SPM at all ages were  $\text{GT}_{1b}$ ,  $\text{GD}_{1b}$ ,  $\text{GD}_{1a}$  and  $\text{GM}_1$ . In addition, the slight decrease in  $\text{GT}_{1b}$  and increase in  $\text{GD}_{1b}$  with age is similar to the observations of Cruz and Gurd (1981).

The nerve ending (synaptic) complex has been speculated as being the putative site for the localization of neuronal gangliosides. This belief arose from the results of studies that have claimed an unusually high concentration of gangliosides in SPM. This hypothesis has been examined in the present study. Our results indicate that the concentration of ganglioside sialic acid of SPM (21.5  $\mu\text{g}$  at 24-days) is very similar to the concentration of ganglioside sialic acid



(20.6  $\mu\text{g}$  sialic acid per mg of microsomal protein at 24 days) in the microsomal membrane fraction. Similarities in the concentration of gangliosides in these two membrane fractions have also been reported by Cruz and Gurd (1981) as well as Skrivanek et al. (1980). These results are in contrast to that of Tettamanti et al. (1980) who have reported concentrations of 13.7 and 27.8  $\mu\text{g}$  for the microsomal and SPM fractions, respectively. The similarities in the concentration of gangliosides in the microsomal and the SPM fraction observed by us as well as other workers suggest that gangliosides are most abundant in neuronal processes but are not exclusively localized at nerve endings. Gangliosides seem to be distributed over a considerable portion of the neuronal surface which would include cell bodies and dendrites as well as nerve endings. In support of this view, Ledeen (1978) has calculated that axon terminals make a relatively small contribution to the total ganglioside content of the cerebral cortex. Antibodies to  $\text{GM}_1$  have revealed intense staining in the granular layer of the rat cerebellum which is composed mainly of neuronal perikarya (De Baeque et al., 1976 and Laev et al., 1978). There is also biochemical evidence that an axolemma-enriched fraction from rat brain has a concentration and pattern of gangliosides roughly equivalent to that of SPM (DeVries and Zmachinski, 1980). Finally, the fact that microsomes which are composed of cell surface membranes as well as internal membranes of brain cells have a relatively high ganglioside

content (Wolfe, 1961, Seminario et al., 1964; Eichberg et al., 1964 and Yohe et al., 1980) as well as similar ganglioside concentrations and patterns to that of SPM (our results; as well as Cruz and Gurd, 1981; Skrivanek et al., 1982) suggest that gangliosides are not localized specifically at nerve endings.

### Brain Regional Gangliosides

Data in the present study provide information about the gangliosides of six different developing brain regions. This is the first developmental report of the concentration and the relative proportions of individual gangliosides in the corpus striatum and the hypothalamus. Furthermore, this is also the first report that examines the gangliosides of various regions within a single brain specimen. Hence, comparisons between the brain regions may be more meaningful because of similar methods of extraction and determination.

In agreement with Vrbaski (1980) and Gombos et al. (1980), we observed a developmentally-related increase in the concentration of ganglioside sialic acid in the cerebellum. As granule cells are the predominant cell type in the cerebellum (Palay and Chan-Palay, 1974), the developmentally related increase in the concentration of ganglioside could be due to the migration and differentiation of granule cells which occurs during the period of study (Altman, 1972; Del Cerro and Snider, 1972; Gombos et al., 1980). Age-related

changes in the relative proportion of individual gangliosides were also observed in the cerebellum. Our finding of comparable proportions of  $GT_{1b}$  and  $GD_{1a}$  at 24 days are similar to the observations of Merat and Dickerson (1973). Since Seyfried et al. (1982) have suggested an enrichment of  $GT_{1a}$  in the Purkinje cells of the cerebellum, our finding of an increase in the relative proportion of  $GT_{1a}$  between 10 and 17 days may relate to the maturation of Purkinje and Golgi neurons which occurs during the same age period (Altman, 1972c).

An increase in the concentration of ganglioside sialic acid in the cerebral cortex observed between 10 and 17 days has also been observed by others. However, De Raveglia et al. (1972) and Vanier et al. (1971) have reported a more dramatic increase during the same age period in the rat cerebral cortex and the rat cerebrum, respectively. The developmentally related decrease in the proportion of  $GT_{1b}$  observed in the cerebral cortex is similar to the observations of Vanier et al. (1971) for the rat cerebrum. In addition, we also observed developmentally related increases in the proportions of  $GD_3$  and  $GM_1$ .  $GM_1$  is elevated in most white matter regions due to its predominance in myelin (Suzuki et al., 1967; Ledeen et al., 1973). Our observed increase in the relative proportion of  $GM_1$  may thus reflect the period of rapid myelination.

The increase in the relative proportion of  $GD_{1b}$  in

the brain stem observed by us is similar to the findings of Yusuf and Dickerson (1978). Furthermore,  $GD_{1b}$  may be an intrinsic constituent of myelin (though not unique) as this ganglioside has been shown to be present in highly purified myelin preparations (Yu and Iqbal, 1979) and has also been shown to be present at higher levels in heavily myelinated CNS (Seyfried et al., 1978). Hence, our observed increase in the relative proportion of  $GD_{1b}$  in the brain stem after 10 days postnatally could reflect the beginning of the period of myelination.

Our studies indicate that the brain stem and cerebellum contain more of the complex ( $GT_1$  and  $GQ_{1b}$ ) ganglioside species than the cerebral cortex. Similar observations have been made in human (Suzuki, 1965) and mouse brain (Seyfried et al., 1978). The similarity in ganglioside distribution observed in the cerebellum and brain stem reflect the similarities in cellular structure and function known to exist in these regions (Shephard, 1974).

The age-related increase in the concentration of gangliosides in the hippocampus in our studies is similar to the observations of Irwin and Irwin (1979). Also, decreases in proportions of the more complex gangliosides  $GT_{1b}$  and  $GQ_{1b}$  and increases in the monosialoganglioside  $GM_1$  have also been observed by Irwin and Irwin (1982) who have attributed these changes to the morphogenetic changes of late neural differentiation in the rat hippocampus.

The comparable relative proportions of  $GD_{1a}$  and  $GT_{1b}$  at 24 days of age in the hypothalamus are similar to the observations of Urban et al. (1979) for adult rat and mouse hypothalamus. In addition, the relative proportion of individual gangliosides in the corpus striatum and the hypothalamus observed at 24 days of age is very similar to that reported by Urban et al. (1979) for adult rat striatum caudate nucleus and hypothalamus.

Our studies clearly demonstrate qualitative and quantitative differences in the concentration and distribution of gangliosides in different brain regions with development. As gangliosides are thought to be enriched in neuronal cell membranes, the regional differences observed by us in terms of ganglioside concentration and relative distribution is probably related to the differences in the number and type of nerve cell membranes.

#### Effect of Maternal Ethanol Consumption on CNS Membranes and Brain Regional Gangliosides

Our studies indicate that the pattern of distribution and synthesis of gangliosides in the three subcellular membrane fractions in the ethanol-exposed pups showed transient abnormalities. Increases in the concentration of ganglioside sialic acid as well as significant alterations in the synthesis and distribution of individual gangliosides were observed in the ethanol-exposed pups. Other pharmacological agents have

been shown to influence the concentration of gangliosides in rat brain and subcellular membrane fractions. (See Brunngraber, 1979 for a review).

Sialic acid is covalently bound to lipids (gangliosides) and proteins (glycoproteins) and is negatively charged (Lehninger, 1975 and Rosenberg and Schengrund, 1976). The sialic acid residues are generally found in terminal positions and project into the cytoplasmic or extracellular space of membranes (Wherrett and McIlwain, 1962; Brunngraber et al., 1967 and Warren, 1976). This stereochemical feature gives sialic acid the potential to interact with the cationic groups of many drugs, metals, hormones and neurotransmitters (Rosenberg and Schengrund, 1976). Such interactions could be affected if sialic acid residues changed spatial orientation as part of either ganglioside or glycoprotein spatial rearrangement responses to ethanol's perturbation of membrane structure (Seeman, 1972).

An explanation for changes in particular ganglioside synthesis and distribution in CNS membranes due to chronic maternal alcohol consumption can at this time only be suggested. It is possible that long term maternal ethanol consumption increases enzymatic destruction of sialic acid by altering the spatial orientation of the sialic acid residues from the membrane surface. It is also possible that chronic maternal ethanol consumption during pregnancy

results in a difference in the timing of morphological events and maturation of cell types during normal CNS development. Volk et al. (1981) have reported a delayed maturation of Purkinje cells during the first two weeks postnatally in the cerebellum of rats that were exposed to ethanol in utero. Similarly, Hammer and Scheibel (1981) have demonstrated via morphological studies that there is a delay in the maturation of pyramidal neurons in the neocortex of rats exposed prenatally to alcohol. Abnormal development of particular neurons or neural cell types could lead to chemical changes which may be revealed in the pattern, distribution and concentration of individual gangliosides. The possibility also exists that ethanol or its metabolites could cause selective destruction of particular cell types or nerve processes. As some gangliosides are now known to be enriched in particular neural cell types (Seyfried et al., 1982), destruction of these cells could result in altered patterns and distribution of gangliosides.

Our results also demonstrate that maternal ethanol consumption resulted in abnormalities both in the synthesis as well as the relative proportion of individual gangliosides in selected brain regions examined. The particular gangliosides that were affected varied with age and brain region. The hippocampus, hypothalamus and corpus striatum were the regions that seemed to be the most affected. The decreased concentration of ganglioside sialic acid as well as altered

synthesis and distribution of individual gangliosides observed in the hippocampus at all ages could reflect cellular damage or arrested development. Barnes and Walker (1981) have reported a 20% loss of dorsal hippocampal pyramidal cells and West et al. (1981) have observed a dramatic alteration in the topographical organization of the hippocampal mossy fiber system in rat. Behavioral studies also indicate that the hippocampus is most affected by prenatal exposure to ethanol. Hyperactivity and deficits in two-way and passive avoidance have been reported (Abel, 1979, 1980; Riley et al., 1979; Bond, 1980; Lochry and Riley, 1980 and Randall and Riley, 1981). Such deficits in behavioral response are consistently observed in animals with hippocampal damage (Kimble, 1975).

It has been hypothesized that gangliosides are involved in receptors for biogenic amines (Wooley and Goomi, 1966; Lapetina et al., 1967 and Dette and Wesemann, 1978). There is considerable evidence available from other laboratories that in utero ethanol exposure has a transient to long term effect on the uptake of CNS neurotransmitters (See Druse, 1981 for a review) as well as on the development of certain neurotransmitter systems (Slotkin et al., 1980). In addition, Michaelis et al. (1980) have found that synaptic membranes exposed to ethanol in vitro results in the disruption of the membrane ganglioside modulation of glutamate binding to its receptor.



The functional significance of the ganglioside abnormalities observed in our study is not clearly understood. In view of the important roles of gangliosides in cell-cell recognition (Irwin, 1974), in brain cell differentiation, recognition, synaptogenesis (Wiegandt, 1968; Irwin, 1974; and Marchase, 1977) and as components of neurotransmitter receptors (Wooley and Goomi, 1966 and Dette and Wesemann, 1978) the abnormalities in the synthesis and distribution of individual gangliosides observed in the present study could be detrimental to normal CNS development and function.

## SUMMARY

The fetal alcohol syndrome (FAS) is a disorder frequently seen in the offspring of chronically alcoholic mothers. The disorder is characterized by pre- and post-natal growth deficiencies, microcephaly, and numerous cranio-facial, skeletal, cardiac, and genital abnormalities. Central nervous system (CNS) dysfunction and irreversible mental retardation are the most serious defects in this syndrome. In order to gain an insight into some of the potential neurochemical causes of mental retardation associated with this syndrome this dissertation examined the effects of chronic maternal ethanol consumption on the glycoproteins and gangliosides of synaptic plasma membranes (SPMs) in the offspring, using the Sprague-Dawley rat as an animal model system. This dissertation also examined the effects of chronic maternal alcohol consumption on SPM glycoproteins in offspring that were cross-fostered to normal chow-fed mothers, since cross-fostering is believed to avoid potential nutritional and nurturing problems and eliminates problems of maintaining ethanol offspring by mothers who may have alcohol- or withdrawal-related hormonal and metabolic disturbances. The hypothesis that the metabolic state of the mother could be responsible for some of the effects of alcohol seen in the offspring was examined by the inclusion of a reverse cross-fostered group.

In addition, since several different types (neuro-

anatomical, neurochemical, and behavioral) of studies suggest that certain brain regions are more susceptible than other areas to the damaging effects of in utero ethanol exposure, this dissertation also examined the effects of chronic maternal ethanol consumption on the gangliosides of selected brain regions in the offspring.

In studies on the synthesis of glycoproteins, [ $^3\text{H}$ ] - or [ $^{14}\text{C}$ ] - fucose served as the glycoprotein precursor and in the case of gangliosides [ $^3\text{H}$ ] - or [ $^{14}\text{C}$ ] - N-acetylmannosamine served as the ganglioside precursor. The isotopes were given intracerebrally as an 18-hour pulse prior to sacrifice of the animals and isolation of the different membrane fractions and brain regions.

Despite the comparable yields of SPMs from ethanol and matched control pups, the ethanol pups demonstrated SPM glycoprotein abnormalities. These abnormalities were most severe in the non-cross-fostered rats whose mothers consumed the original (Lieber-DeCarli) liquid diet, although abnormalities were also found in the cross-fostered offspring of rats whose mothers received the revised (21% protein) liquid diets. Interestingly, the reverse cross-fostered group of rats did not show SPM glycoprotein abnormalities, thereby demonstrating that the SPM glycoprotein abnormalities were only present in the biologic offspring of "alcoholic rats". It appears that chronic maternal ethanol consumption has a severe effect on the synthesis of SPM glycoproteins

in developing offspring and this abnormality seems to be augmented by poor nutrition, although the nutritional status is not solely responsible for these effects. In addition, the abnormalities are primarily due to a prenatal effect and not exclusively dependent on the status (hormonal, metabolic, etc.) of the lactating dams.

Studies on the gangliosides of microsomal, synaptosomal, and SPM fractions revealed that gangliosides are most abundant in neuronal processes but are not exclusively localized at nerve endings. In addition, the pattern of distribution and synthesis of gangliosides in the three subcellular membrane fractions showed transient abnormalities.

Studies on gangliosides in selected brain regions revealed that the concentration as well as the relative proportion of individual gangliosides differed for each region as well as changed with development. The study also demonstrated that maternal ethanol consumption resulted in abnormalities both in the synthesis as well as the relative proportion of individual gangliosides in selected brain regions in the offspring. The particular gangliosides that were affected varied with age and brain region. The hippocampus, hypothalamus, and corpus striatum were the regions that were most affected by in utero exposure to ethanol.

The functional significance of the glycoprotein and ganglioside abnormalities observed in this study is not clearly understood. However, in view of the postulated

important roles of glycoproteins and gangliosides in cell-cell recognition, adhesion, and synaptic connectivity, and as components of neurotransmitter receptors, the abnormalities observed in the synthesis and distribution of glycoproteins and gangliosides could be detrimental to normal CNS development and function.

The research reported represents the first account of the effects of chronic maternal ethanol consumption on the synthesis of SPM fucosylated glycoproteins in the offspring. It also represents the first report of the effects on the synthesis and distribution of individual gangliosides of subcellular membrane fractions and selected brain regions in offspring.

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## APPENDIX

Figure A-1: Distribution of fucose-derived radioactivity in SPMs from 10 day-old control and ethanol pups.

Figure A depicts the distribution of radioactivity in dpm and figure B depicts the distribution of %dpm -- (dpm in a given fraction/total gel dpm) X 100. The scale for the left ordinate in A represents the [<sup>3</sup>H] and [<sup>14</sup>C] dpm.

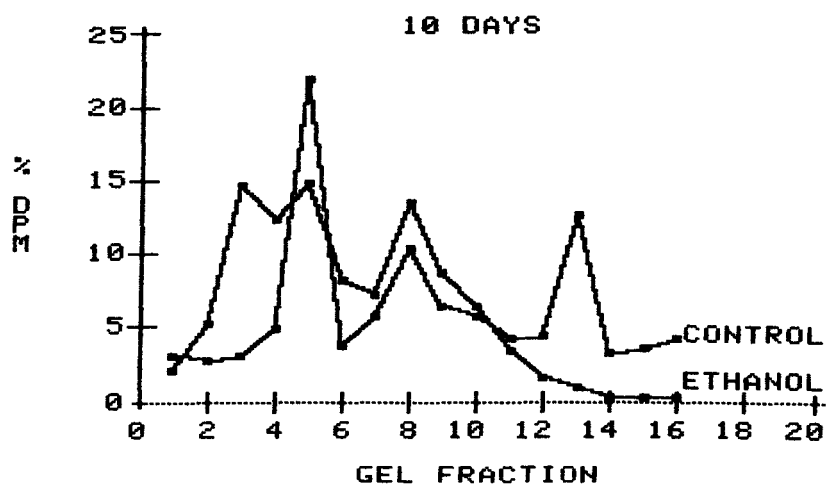
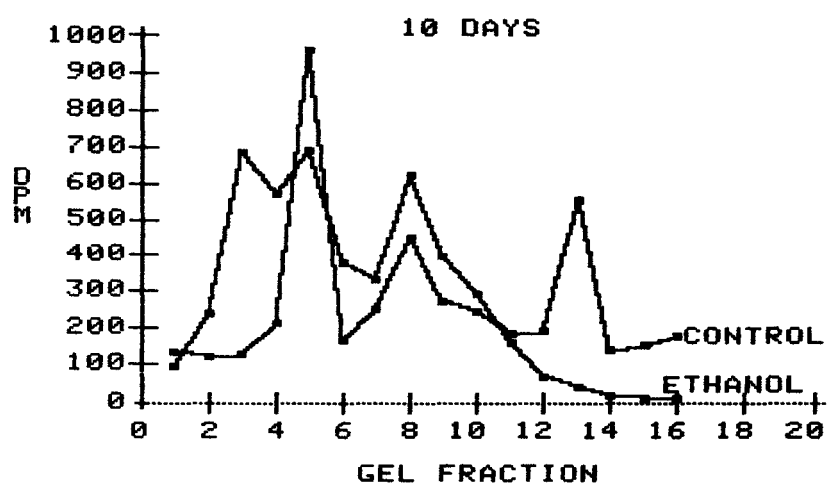




Figure A-2: Distribution of fucose-derived radioactivity in SPMs from 17 day-old control and ethanol pups.

Figure A depicts the distribution of radioactivity in dpm and figure B depicts the distribution of %dpm -- (dpm in a given fraction/total gel dpm) X 100. The scales for the left and right ordinates in A represent the [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ] dpm.

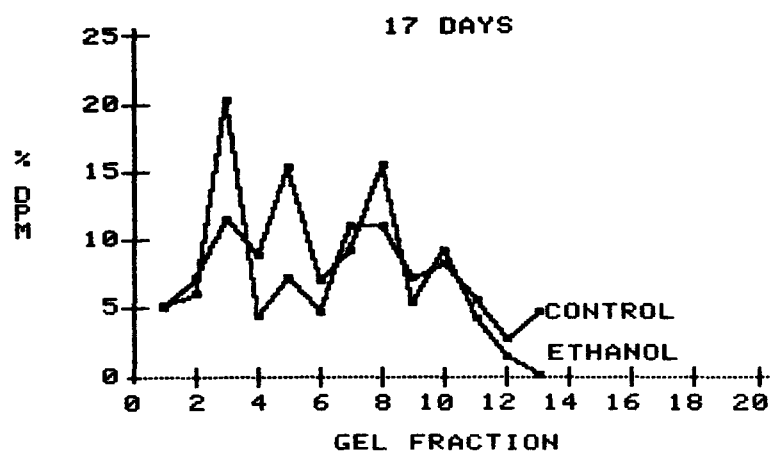
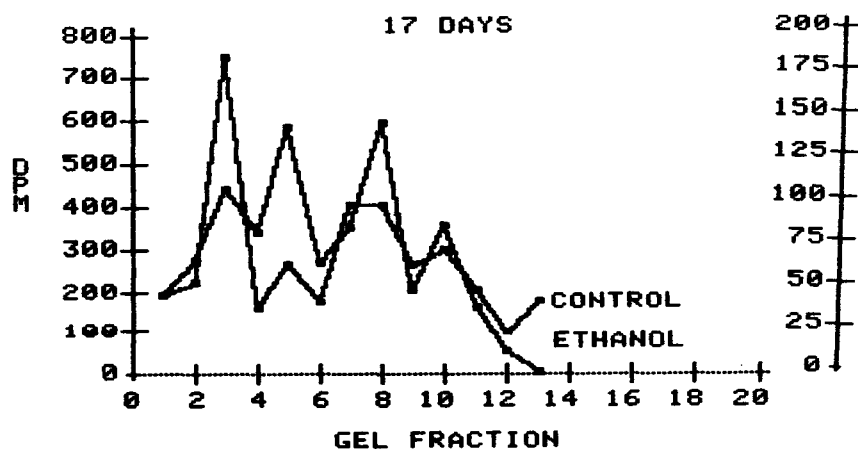


Figure A-3: Distribution of fucose-derived radioactivity in SPMs from 24 day-old control and ethanol pups.

Figure A depicts the distribution of radioactivity in dpm and figure B depicts the distribution of %dpm -- (dpm in a given fraction/total gel dpm) X 100. The scale for the left ordinate in A represents the [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ]dpm.

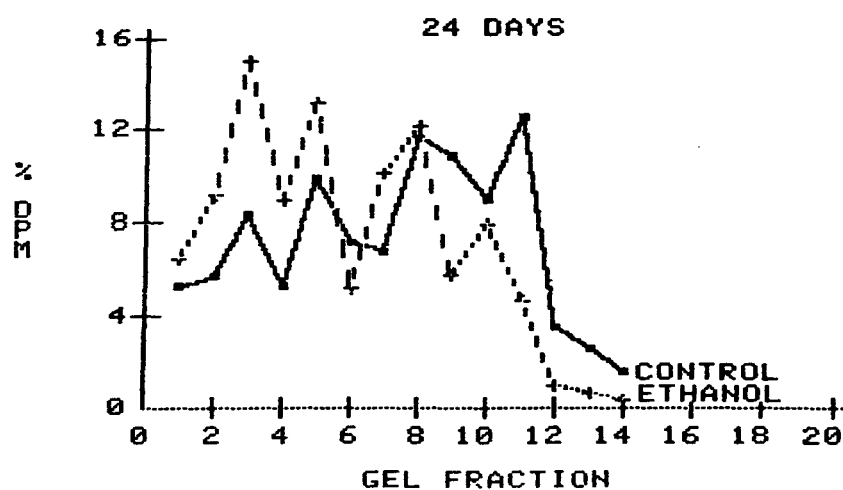
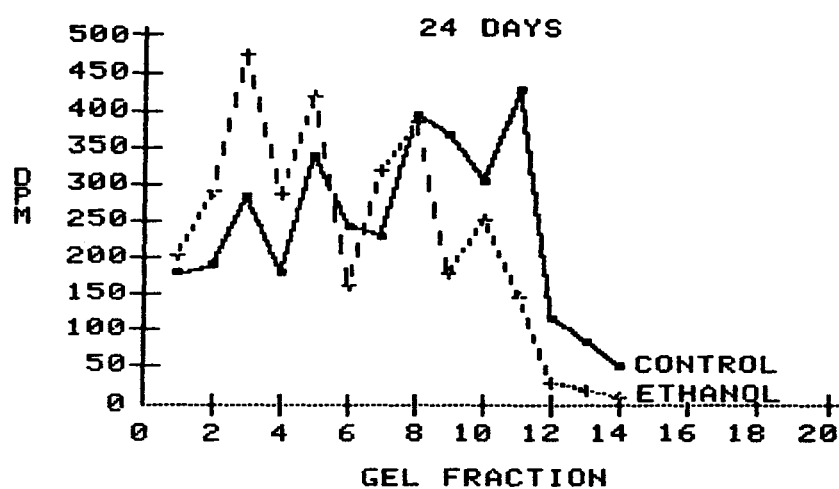


Figure A-4: Distribution of fucose-derived radioactivity in SPMs from 10 day-old control and ethanol cross-fostered pups.

Figure A depicts the distribution of radioactivity in dpm and figure B depicts the distribution of %dpm -- (dpm in a given fraction/total gel dpm) X 100. The scale for the left and right ordinates in A represent the [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ] dpm.

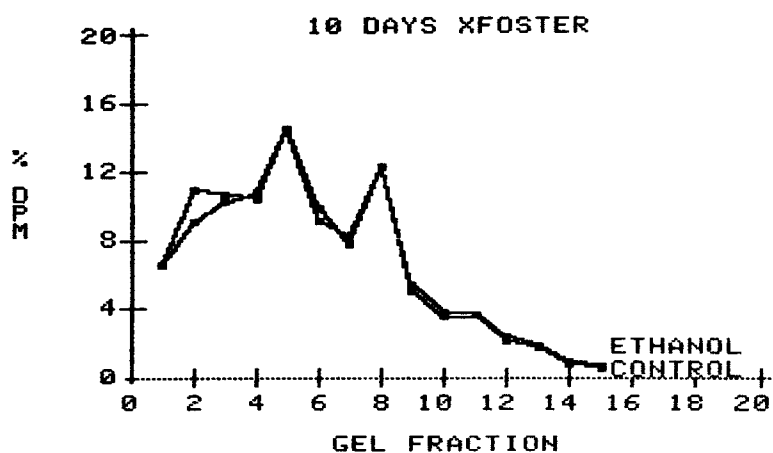
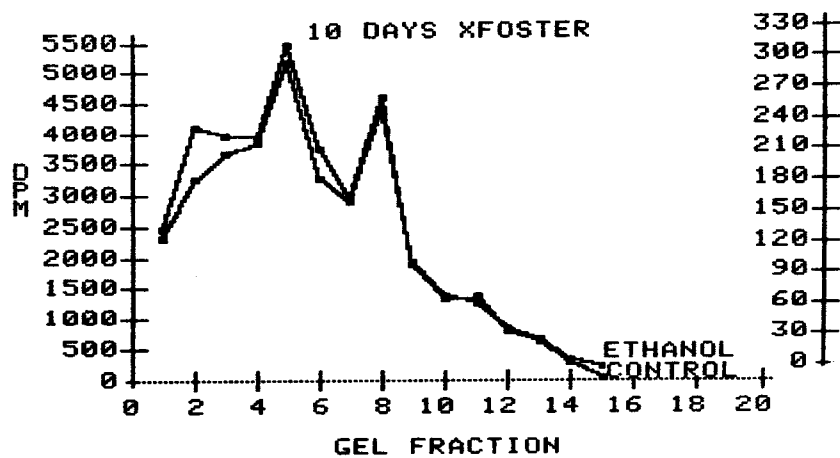


Figure A-5: Distribution of fucose-derived radioactivity in SPMs from 17 day-old control and ethanol cross-fostered pups.

Figure A depicts the distribution of radioactivity in dpm and figure B depicts the distribution of %dpm -- (dpm in a given fraction/total gel dpm) X 100. The scales for the left and right ordinates in A represent the [<sup>3</sup>H] and [<sup>14</sup>C] dpm.

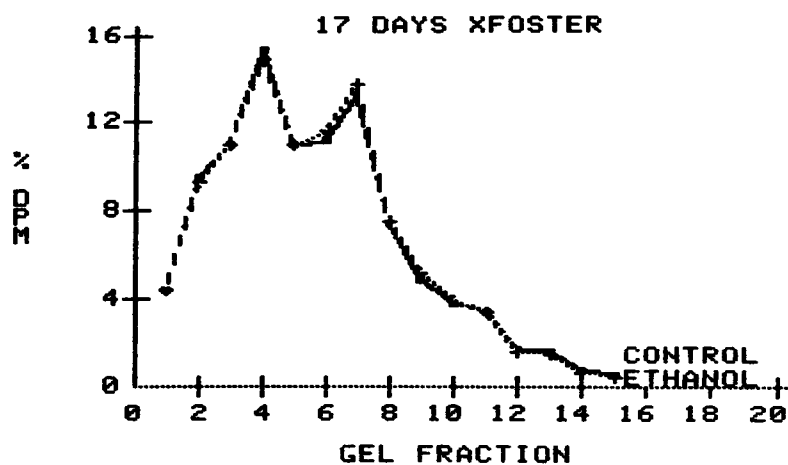
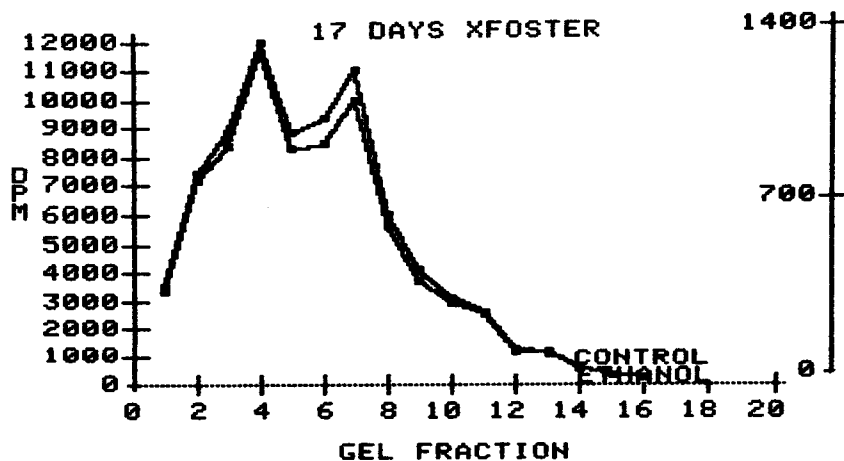




Figure A-6: Distribution of fucose-derived radioactivity in SPMs from 24 day-old control and ethanol cross-fostered pups.

Figure A depicts the distribution of radioactivity in dpm and figure B depicts the distribution of %dpm -- (dpm in a given fraction/total gel dpm) X 100. The scales for the left and right ordinates in A represent the [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ] dpm.

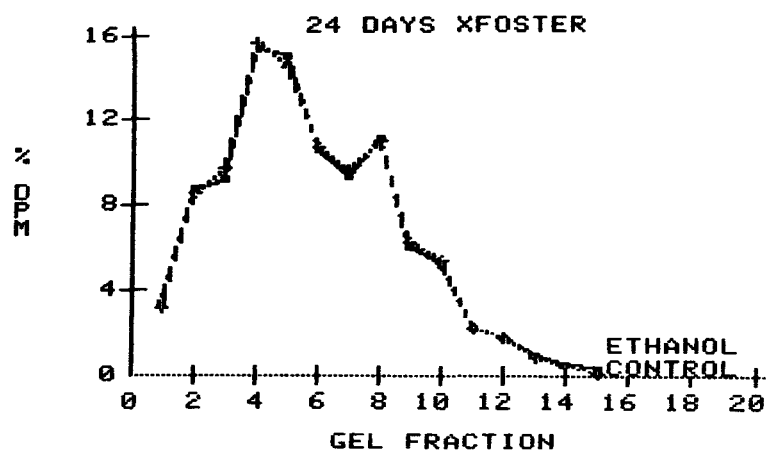
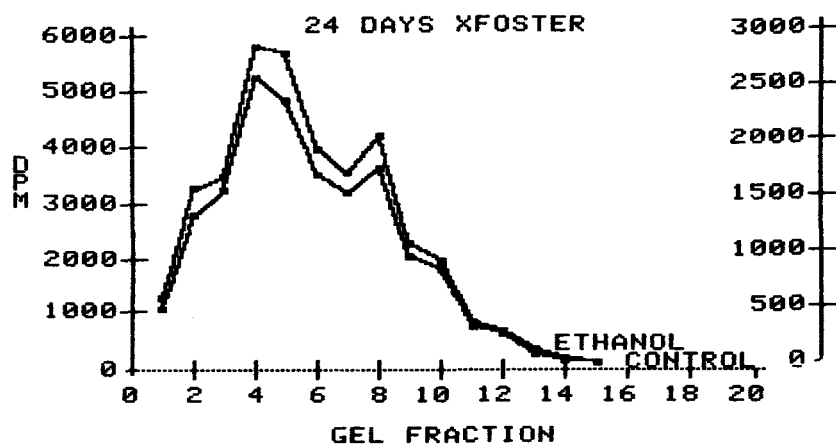


Figure A-7: Distribution of fucose-derived radioactivity in SPMs from 10 day-old control and ethanol reverse-cross-fostered pups.

Figure A depicts the distribution of radioactivity in dpm and figure B depicts the distribution of %dpm -- (dpm in a given fraction/total gel dpm) X 100. The scales for the left and right ordinates in A represent the [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ] dpm.

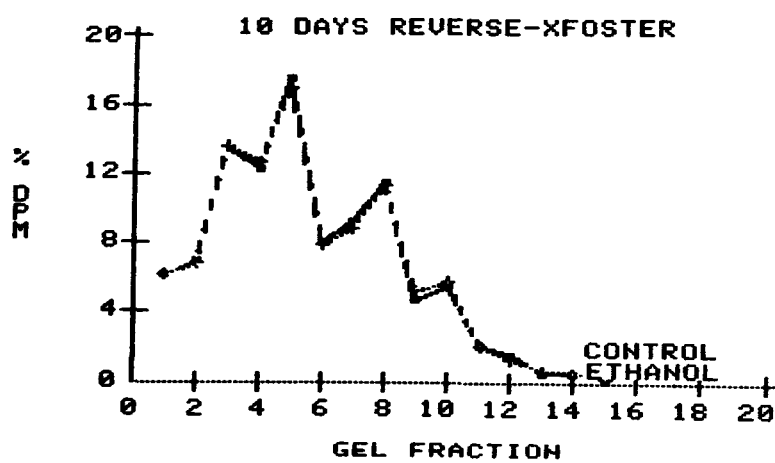
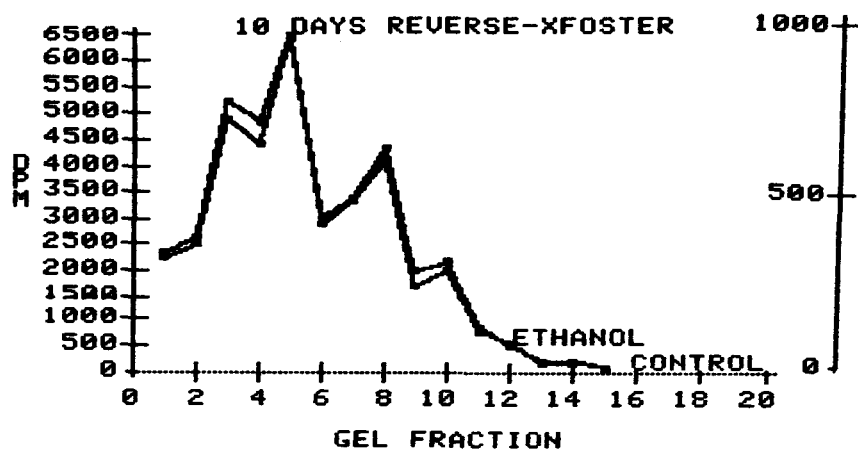


Figure A-8: Distribution of fucose-derived radioactivity in SPMs from 17 day-old control and ethanol reverse-cross-fostered pups.

Figure A depicts the distribution of radioactivity in dpm and figure B depicts the distribution of %dpm -- (dpm in a given fraction/total gel dpm) X 100. The scales for the left and right ordinates represent the [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ] dpm in figure A.

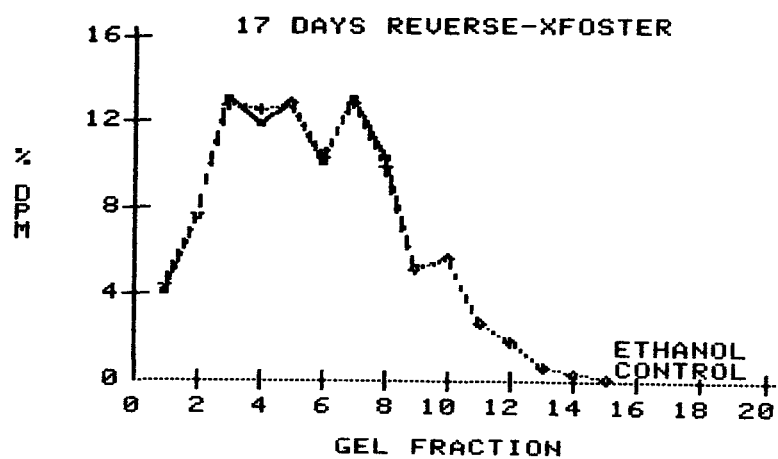
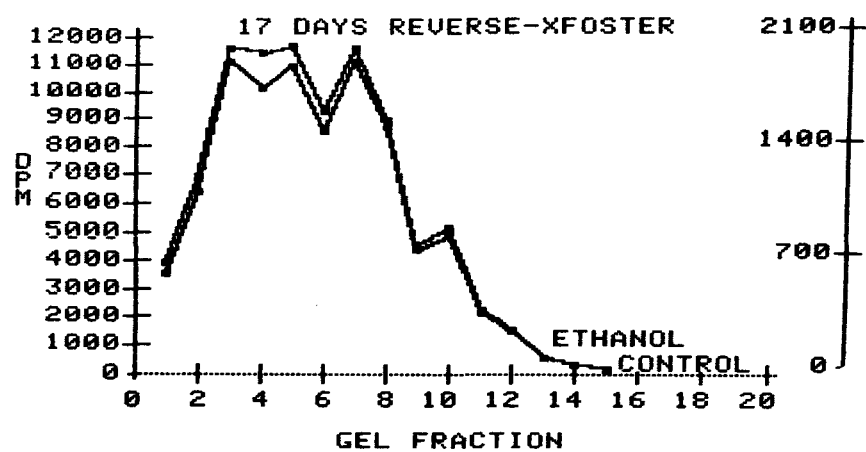


Figure A-9: Distribution of fucose-derived radioactivity in SPMs from 24 day-old control and ethanol reverse-cross-fostered pups.

Figure A depicts the distribution of radioactivity in dpm and figure B depicts the distribution of %dpm -- (dpm in a given fraction/total gel dpm) X 100. The scales for the left and right ordinates in A represent the [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ] dpm.

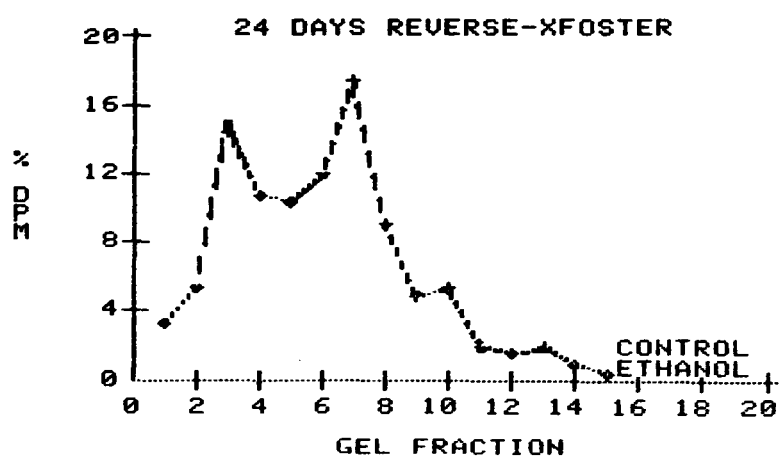
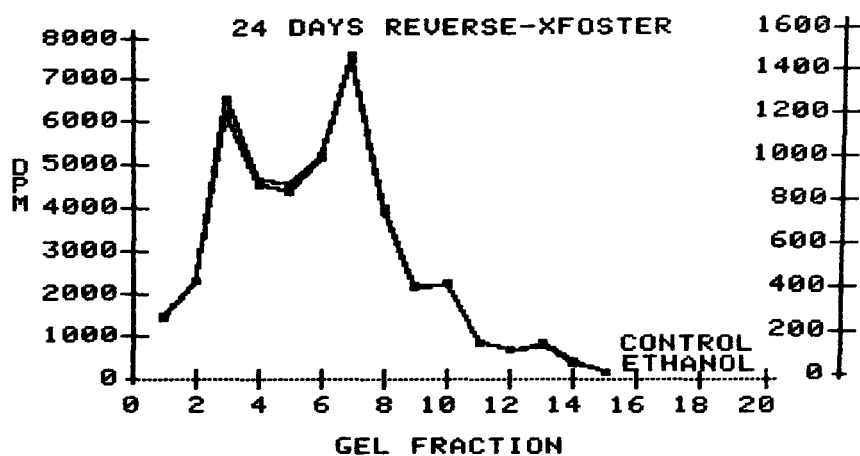




TABLE A-I : Effects of Maternal Ethanol Consumption on the % Distribution of Sialic Acid Among Individual Gangliosides of Cerebral Cortices in Offspring.

Gangliosides were extracted from the cerebral cortices of individual control (C) and ethanol (E) pups as described in "Materials and Methods" at 10, 17, and 24 days of age. An aliquot of the extracted ganglioside sialic acid was spotted as a 1.5 cm streak on HPTLC plates (10X10 cm) and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by the resorcinol spray (Svennerholm, 1957). Identification of the ganglioside bands was achieved by comparing their chromatographic mobilities with the standards. The distribution of individual gangliosides was determined directly by densitometric scanning of the resorcinol positive spots, by the procedure of Ando et al. (1978). The percent distribution of individual gangliosides in each sample was determined by a 3390A integrator (Hewlett-Packard). Results are expressed as the percentage of total ganglioside sialic acid. Gangliosides are named according to SVENNERHOLM's nomenclature.

Each value represents the mean  $\pm$  the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

(--) indicates that these gangliosides were not detected.

TABLE A-I  
Effects of Maternal Ethanol Consumption  
on % Distribution of Sialic Acid among Individual Gangliosides of Cerebral Cortices in Offspring

Ganglioside	10 Days		17 Days		24 Days	
	Control	Ethanol	Control	Ethanol	Control	Ethanol
GQ1b	4.0±1.7	7.7±2.0*	5.4±1.5	2.6±2.9	1.1±0.5	0.9±0.1
GT1b	22.5±1.0	19.8±0.7*	18.9±1.8	15.2±3.4	11.8±2.2	15.0±1.1
GT1a	2.1±0.4	2.5±0.7	2.0±1.0	2.5±0.6	2.0±0.9	1.8±0.4
GD1b	10.8±1.2	9.9±0.4	11.7±1.6	13.7±1.4*	11.2±2.8	13.3±0.2
GD2	7.9±2.5	8.9±1.7	7.0±0.5	6.0±1.3	42.0±2.4	41.2±1.1
GD1a	38.4±4.7	36.9±2.3	35.6±3.9	38.9±6.1		
GD3	3.3±0.8	3.6±0.9	4.9±1.8	5.9±0.6	7.2±1.9	5.6±0.3
GM1	11.0±0.6	10.6±1.1	14.5±2.8	15.4±2.6	24.8±3.5	22.4±2.8
GM3	---	---	---	---	---	---
GM4	---	---	---	---	---	---

Each value represents the mean ± the standard deviation of six animals.

A \* indicates that values from control and ethanol pups from the same groups are different at  $p < 0.05$ .

TABLE A-II: Effects of Maternal Ethanol Consumption on the % Distribution of Sialic Acid Among Individual Gangliosides of Cerebella in Offspring.

Gangliosides were extracted from the cerebella of individual control (C) and ethanol (E) pups as described in "Materials and Methods" at 10, 17, and 24 days of age. An aliquot of the extracted ganglioside sialic acid was spotted as a 1.5 cm streak on HPTLC plates (10X10 cm) and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by the resorcinol spray (Svennerholm, 1957). Identification of the ganglioside bands was achieved by comparing their chromatographic mobilities with the standards. The distribution of individual gangliosides was determined directly by densitometric scanning of the resorcinol positive spots, by the procedure of Ando et al. (1978). The percent distribution of individual gangliosides in each sample was determined by a 3390A integrator (Hewlett-Packard). Results are expressed as the percentage of total ganglioside sialic acid. Gangliosides are named according to SVENNERHOLM's nomenclature.

Each value represents the mean  $\pm$  the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

(--) indicates that these gangliosides were not detected.

TABLE A-II

## Effects of Maternal Ethanol Consumption

on % Distribution of Sialic Acid among Individual Gangliosides of Cerebella in Offspring

Ganglioside	10 Days		17 Days		24 Days	
	Control	Ethanol	Control	Ethanol	Control	Ethanol
GQ1b	5.0±0.8	6.0±0.1*	6.0±1.1	6.7±0.9	6.2±1.2	5.9±0.4
GT1b	28.5±2.6	26.4±1.0	26.7±2.0	28.1±0.9	23.5±2.6	24.6±1.9
GT1a	2.1±0.3	2.7±1.0	6.7±0.2	5.8±0.9	5.7±0.4	6.2±0.7
GD1b	12.5±0.7	12.5±1.0	11.5±1.3	10.4±1.8	9.4±1.1	8.8±0.3
GD2	4.4±1.0	4.3±2.2	7.0±2.9	5.8±1.6	8.5±1.6	7.7±2.6
GD1a	30.7±1.3	29.2±1.9	24.0±2.3	25.0±1.8	25.7±1.6	28.8±2.4
GD3	4.4±1.0	5.5±0.7	6.8±0.5	6.6±0.3	4.9±0.9	5.1±0.8
GM1	7.5±0.9	8.1±1.2	5.9±0.3	5.9±0.4	6.8±0.8	6.1±0.7
GM3	2.0±0.4	2.9±1.2	2.8±1.4	2.9±1.3	3.7±1.7	4.4±1.7
GM4	2.9±0.3	2.6±0.6	2.7±0.7	2.6±0.9	3.5±1.6	2.4±0.8

Each value represents the mean ± the standard deviation of six animals.

A \* indicates that values from control and ethanol pups from the same groups are different at  $p < 0.05$ .

TABLE A-III: Effects of Maternal Ethanol Consumption on the % Distribution of Sialic Acid Among Individual Gangliosides of Brain Stem in Offspring.

Gangliosides were extracted from the brain stem of individual control (C) and ethanol (E) pups as described in "Materials and Methods" at 10, 17, and 24 days of age. An aliquot of the extracted ganglioside sialic acid was spotted as a 1.5 cm streak on HPTLC plates (10X10 cm) and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by the resorcinol spray (Svennerholm, 1957). Identification of the ganglioside bands was achieved by comparing their chromatographic mobilities with the standards. The distribution of individual gangliosides was determined directly by densitometric scanning of the resorcinol positive spots, by the procedure of Ando et al. (1978). The percent distribution of individual gangliosides in each sample was determined by a 3390A integrator (Hewlett-Packard). Results are expressed as the percentage of total ganglioside sialic acid. Gangliosides are named according to SVENNERHOLM's nomenclature.

Each value represents the mean  $\pm$  the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

(--) indicates that these gangliosides were not detected.

TABLE A-III

## Effects of Maternal Ethanol Consumption

on % Distribution of Sialic Acid among Individual Gangliosides of Brain Stem in Offspring

Ganglioside	10 Days		17 Days		24 Days	
	Control	Ethanol	Control	Ethanol	Control	Ethanol
GQ1b	9.4±1.3	10.2±1.4	4.8±0.8	4.8±0.6	N.D.	N.D.
GT1b	28.5±3.8	29.0±1.2	21.7±2.1	23.1±4.4	N.D.	N.D.
GT1a	4.9±3.7	2.2±0.7	9.5±2.4	11.5±1.4	N.D.	N.D.
GD1b	13.9±1.5	15.3±0.7	21.2±7.0	23.4±5.6	N.D.	N.D.
GD2	---	---	---	---	N.D.	N.D.
GD1a	27.4±1.1	25.4±2.5	27.3±2.4	27.9±5.8	N.D.	N.D.
GD3	2.7±1.4	3.0±1.5	4.2±1.4	4.8±0.7	N.D.	N.D.
GM1	6.8±1.5	7.5±0.7	9.5±1.4	9.2±1.3	N.D.	N.D.
GM3	5.1±0.4	5.7±2.0	2.5±2.1	1.3±0.9	N.D.	N.D.
GM4	2.9±0.6	2.5±0.5	2.9±1.3	2.9±0.7	N.D.	N.D.

Each value represents the mean ± the standard deviation of six animals.

A \* indicates that values from control and ethanol pups from the same groups are different at  $p < 0.05$ .

N.D. indicates that data from these animals were not determined.

TABLE A-IV: Effects of Maternal Ethanol Consumption on the % Distribution of Sialic Acid Among Individual Gangliosides of Hippocampus in Offspring.

Gangliosides were extracted from the hippocampus of individual control (C) and ethanol (E) pups as described in "Materials and Methods" at 10, 17, and 24 days of age. An aliquot of the extracted ganglioside sialic acid was spotted as a 1.5 cm streak on HPTLC plates (10X10 cm) and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by the resorcinol spray (Svennerholm, 1957). Identification of the ganglioside bands was achieved by comparing their chromatographic mobilities with the standards. The distribution of individual gangliosides was determined directly by densitometric scanning of the resorcinol positive spots, by the procedure of Ando et al. (1978). The percent distribution of individual gangliosides in each sample was determined by a 3390A integrator (Hewlett-Packard). Results are expressed as the percentage of total ganglioside sialic acid. Gangliosides are named according to SVENNERHOLM's nomenclature.

Each value represents the mean  $\pm$  the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

(--) indicates that these gangliosides were not detected.

TABLE A-IV

Effects of Maternal Ethanol Consumption  
on % Distribution of Sialic Acid among Individual Gangliosides of Hippocampus in Offspring

Ganglioside	10 Days		17 Days		24 Days	
	Control	Ethanol	Control	Ethanol	Control	Ethanol
GQ1b	11.7±3.0	9.3±4.2	10.1±1.4	10.2±1.4	5.8±1.2	9.3±2.1*
GT1b	24.9±4.8	33.0±2.6*	29.9±3.5	29.2±2.3	23.2±2.9	22.3±1.2
GT1a	---	---	3.2±0.8	3.1±0.5	4.3±0.3	5.3±0.4*
GD1b	11.8±3.4	10.8±1.3	12.1±1.5	14.1±0.6*	12.0±0.7	10.9±0.9*
GD2	---	---	---	---	---	---
GD1a	36.0±5.9	36.0±3.1	33.9±2.2	33.6±1.6	36.2±2.4	33.2±3.1
GD3	4.2±0.6	4.0±0.5	3.2±1.8	2.4±0.5	5.5±0.9	6.8±1.4
GM1	9.4±0.4	7.0±0.5*	7.5±1.3	7.6±0.4	12.9±1.0	11.2±1.7
GM3	---	---	---	---	---	---
GM4	---	---	---	---	---	---

Each value represents the mean ± the standard deviation of six animals.

A \* indicates that values from control and ethanol pups from the same groups are different at  $p < 0.05$ .



TABLE A-V: Effects of Maternal Ethanol Consumption on the % Distribution of Sialic Acid Among Individual Gangliosides of Corpus Striata in Offspring.

Gangliosides were extracted from the corpus striata of individual control (C) and ethanol (E) pups as described in "Materials and Methods" at 10, 17, and 24 days of age. An aliquot of the extracted ganglioside sialic acid was spotted as a 1.5 cm streak on HPTLC plates (10X10 cm) and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by the resorcinol spray (Svennerholm, 1957). Identification of the ganglioside bands was achieved by comparing their chromatographic mobilities with the standards. The distribution of individual gangliosides was determined directly by densitometric scanning of the resorcinol positive spots, by the procedure of Ando et al. (1978). The percent distribution of individual gangliosides in each sample was determined by a 3390A integrator (Hewlett-Packard). Results are expressed as the percentage of total ganglioside sialic acid. Gangliosides are named according to SVENNERHOLM's nomenclature.

Each value represents the mean  $\pm$  the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

(--) indicates that these gangliosides were not detected.

TABLE A-V

Effects of Maternal Ethanol Consumption  
on % Distribution of Sialic Acid among Individual Gangliosides of Corpus Striata in Offspring

Ganglioside	10 Days		17 Days		24 Days	
	Control	Ethanol	Control	Ethanol	Control	Ethanol
GQ1b	6.3±1.1	5.6±0.5	4.0±0.8	3.4±0.6	4.9±1.3	5.2±0.5
GT1b	22.2±2.0	26.1±2.6*	23.1±1.4	23.9±2.5	21.8±1.3	19.8±1.3*
GT1a	1.5±0.5	0.9±0.4			2.0±0.4	1.3±0.6*
GD1b	12.0±1.1	9.3±0.9*	11.8±1.0	10.9±0.3	14.0±1.4	11.9±0.9*
GD2	---	---	---	---	---	---
GD1a	38.7±2.3	40.9±2.4	40.1±1.3	40.9±3.6	38.3±2.8	42.8±2.1*
GD3	5.8±0.6	5.3±1.0	3.5±1.6	3.7±1.0	3.8±1.7	2.6±0.9
GM1	13.6±1.6	11.9±0.9*	16.1±1.5	17.3±2.5	15.4±2.5	16.4±1.2
GM3	---	---	---	---	---	---
GM4	---	---	---	---	---	---

Each value represents the mean ± the standard deviation of six animals.

A \* indicates that values from control and ethanol pups from the same groups are different at  $p < 0.05$ .

TABLE A-VI: Effects of Maternal Ethanol Consumption on the % Distribution of Sialic Acid Among Individual Gangliosides of Hypothalamus in Offspring.

Gangliosides were extracted from the hypothalamus of individual control (C) and ethanol (E) pups as described in "Materials and Methods" at 10, 17, and 24 days of age. An aliquot of the extracted ganglioside sialic acid was spotted as a 1.5 cm streak on HPTLC plates (10X10 cm) and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by the resorcinol spray (Svennerholm, 1957). Identification of the ganglioside bands was achieved by comparing their chromatographic mobilities with the standards. The distribution of individual gangliosides was determined directly by densitometric scanning of the resorcinol positive spots, by the procedure of Ando et al. (1978). The percent distribution of individual gangliosides in each sample was determined by a 3390A integrator (Hewlett-Packard). Results are expressed as the percentage of total ganglioside sialic acid. Gangliosides are named according to SVENNERHOLM's nomenclature.

Each value represents the mean  $\pm$  the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

(--) indicates that these gangliosides were not detected.

TABLE XVI

Effects of Maternal Ethanol Consumption  
on % Distribution of Sialic Acid among Individual Gangliosides of Hypothalamus in Offspring

Ganglioside	10 Days		17 Days		24 Days	
	Control	Ethanol	Control	Ethanol	Control	Ethanol
GQ1b	8.6±2.0	8.6±2.0	7.5±0.5	6.5±0.9	10.1±1.2	11.3±1.6
GT1b	29.0±0.9	29.8±1.4	27.9±0.8	27.4±1.7	29.9±2.2	27.7±0.2
GT1a	1.3±0.7	1.2±0.7	2.3±0.3	2.1±0.2	2.5±0.4	3.1±0.3*
GD1b	12.7±1.5	13.1±1.0	16.2±0.5	16.6±0.5	16.0±0.2	16.8±0.2*
GD2	---	---	---	---	---	---
GD1a	38.1±2.5	35.8±0.9	31.3±2.0	33.7±1.6*	28.1±1.4	28.5±1.0
GD3	2.2±1.0	2.4±1.3	3.7±1.3	3.6±0.6	5.3±0.7	4.3±0.2*
GM1	7.5±0.7	8.5±1.9	10.9±1.6	9.2±1.1*	8.2±0.3	8.4±0.6
GM3	---	---	---	---	---	---
GM4	---	---	---	---	---	---

Each value represents the mean ± the standard deviation of six animals.

A \* indicates that values from control and ethanol pups from the same groups are different at  $p < 0.05$ .

TABLE A-VII : Effects of Maternal Ethanol Consumption on The % distribution of Sialic Acid among Individual Radiolabeled Gangliosides of Cerebral Cortex in Offspring.

Radiolabeled gangliosides were extracted from the cerebral cortex of control (C) and ethanol (E) pups at 10, 17 and 24 days of age. A mixture of radiolabeled gangliosides from a control (C) and ethanol (E) rat of the same age was spotted on a HPTLC plate and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by exposing the plate to  $I_2$  vapor in a glass tank. Individual gangliosides were scraped from the plate and solubilized in water and 10 ml of Aquasol. Radioactivity was determined by means of a liquid scintillation counter. Approximately 20,000 [ $^3H$ ] dpm (control) and 5,000 [ $^{14}C$ ] dpm (ethanol) were recovered from each lane. Results are expressed as the percentage of total DPM of ganglioside sialic acid DPM.

Each value represents the mean  $\pm$  the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

(--) indicates that these gangliosides were not detected.

TABLE A-VII

Effects of Maternal Ethanol Consumption  
on % Distribution of Sialic Acid among Individual Radiolabeled Gangliosides of Cerebral Cortices in Offspring

Ganglioside	10 Days		17 Days		24 Days	
	Control	Ethanol	Control	Ethanol	Control	Ethanol
GQ1b	7.6±1.0	3.8±1.5*	4.7±1.2	5.6±1.5	3.5±1.2	2.6±1.0
GT1b	15.7±2.6	11.9±4.6	15.5±3.1	16.1±4.2	13.3±2.8	16.7±3.0
GT1a			2.8±1.0	1.7±0.6		
GD1b	11.5±2.3	10.2±0.4	12.0±2.2	9.0±2.1*	15.1±1.3	15.9±0.6
GD2	8.4±1.4	9.8±1.3	9.0±1.4	6.7±1.1*	40.5±1.9	43.4±3.9
GD1a	35.2±2.0	41.6±3.7*	36.9±0.7	37.0±1.5		
GD3	5.2±0.2	3.2±0.1*	4.2±1.0	2.5±0.6*	9.3±1.5	6.1±0.2*
GM1	16.5±0.9	19.5±3.5	15.1±0.4	21.2±1.1*	18.3±1.1	15.3±1.0*
GM3	---	---	---	---	---	---
GM4	---	---	---	---	---	---

Each value represents the mean ± the standard deviation of six animals.

A \* indicates that values from control and ethanol pups from the same groups are different at  $p < 0.05$ .

TABLE A-VIII: Effects of Maternal Ethanol Consumption on The % distribution of Sialic Acid among Individual Radiolabeled Gangliosides of Cerebella in Offspring.

Radiolabeled gangliosides were extracted from the cerebella of control (C) and ethanol (E) pups at 10, 17 and 24 days of age. A mixture of radiolabeled gangliosides from a control (C) and ethanol (E) rat of the same age was spotted on a HPTLC plate and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by exposing the plate to I<sub>2</sub> vapor in a glass tank. Individual gangliosides were scraped from the plate and solubilized in water and 10 ml of Aquasol. Radioactivity was determined by means of a liquid scintillation counter. Approximately 15,000 [<sup>3</sup>H] dpm (control) and 4,000 [<sup>14</sup>C] dpm (ethanol) were recovered from each lane. Results are expressed as the percentage of total DPM of ganglioside sialic acid DPM.

Each value represents the mean  $\pm$  the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

(-- ) indicates that these gangliosides were not detected.

TABLE A-VIII

Effects of Maternal Ethanol Consumption  
on % Distribution of Sialic Acid among Individual Radiolabeled Gangliosides of Offspring

Ganglioside	10 Days		17 Days		24 Days	
	Control	Ethanol	Control	Ethanol	Control	Ethanol
GQ1b	7.3±0.9	8.0±1.4	4.8±0.6	4.9±1.0	6.0±0.9	6.7±1.2
GT1b	23.9±1.1	24.7±1.1	29.8±1.3	32.3±2.1	25.1±1.3	27.1±2.2*
GT1a	3.4±0.6	3.6±0.4			5.1±0.5	5.9±0.6*
GD1b	13.9±1.3	11.6±0.6*	12.4±1.2	12.2±1.1	9.2±2.4	8.1±1.6
GD2	28.4±2.9	28.2±3.4	9.0±0.6	6.5±0.9*	6.2±2.4	5.5±2.7
GD1a			27.7±0.5	30.0±4.3	27.4±3.2	29.2±2.1
GD3	12.1±0.8	14.2±1.8*	7.1±0.9	4.7±0.6*	4.8±0.6	4.9±0.6
GM1			6.9±1.2	5.9±1.4	8.7±0.8	5.9±0.4*
GM3	6.3±1.0	6.4±2.3	1.9±0.5	2.6±0.5*	4.4±0.8	3.0±1.4
GM4	4.0±0.7	4.2±0.7	0.5±0.2	0.4±0.1	3.3±1.1	3.3±1.2

Each value represents the mean ± the standard deviation of six animals.

A \* indicates that values from control and ethanol pups from the same groups are different at  $p < 0.05$ .



TABLE A-IX : Effects of Maternal Ethanol Consumption on The % distribution of Sialic Acid among Individual Radiolabeled Gangliosides of Brain Stem.

Radiolabeled gangliosides were extracted from the brain stem of control (C) and ethanol (E) pups at 10, 17 and 24 days of age. A mixture of radiolabeled gangliosides from a control (C) and ethanol (E) rat of the same age was spotted on a HPTLC plate and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by exposing the plate to I<sub>2</sub> vapor in a glass tank. Individual gangliosides were scraped from the plate and solubilized in water and 10 ml of Aquasol. Radioactivity was determined by means of a liquid scintillation counter. Approximately 50,000 [<sup>3</sup>H] dpm (control) and 10,000 [<sup>14</sup>C] dpm (ethanol) were recovered from each lane. Results are expressed as the percentage of total DPM of ganglioside sialic acid DPM.

Each value represents the mean  $\pm$  the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

(--) indicates that these gangliosides were not detected.

TABLE A-IX

## Effects of Maternal Ethanol Consumption

on % Distribution of Sialic Acid among Individual Radiolabeled Gangliosides of Brain Stem in Offspring

Ganglioside	10 Days		17 Days		24 Days	
	Control	Ethanol	Control	Ethanol	Control	Ethanol
GQ1b	7.5±0.6	6.6±1.7	6.0±0.7	6.6±0.4	5.9±1.6	7.6±1.9
GT1b	29.6±2.1	29.9±2.1	28.3±3.0	32.7±2.9*	33.5±0.7	35.2±2.5
GT1a						
GD1b	14.0±1.1	13.5±0.5	18.2±3.1	16.3±1.3	10.8±1.5	13.0±2.3
GD2	---	---	---	---	---	---
GD1a	29.9±2.0	29.7±4.5	24.8±2.4	25.0±0.5	5.2±0.7	25.6±1.8
GD3	1.5±0.6	2.1±0.2	5.5±0.6	4.9±1.0	4.9±0.8	4.2±1.1
GM1	10.0±1.4	11.9±2.3	10.2±2.4	8.5±1.8	14.6±1.1	11.0±1.2*
GM3	3.5±0.4	2.1±0.8*	4.1±0.6	3.6±0.3	3.1±1.0	2.1±0.6
GM4	3.4±1.1	3.5±0.8	2.9±0.7	2.5±0.4	2.1±0.6	1.3±0.6

Each value represents the mean ± the standard deviation of six animals.

A \* indicates that values from control and ethanol pups from the same groups are different at  $p < 0.05$ .

TABLE A-X : Effects of Maternal Ethanol Consumption on The % distribution of Sialic Acid among Individual Radiolabeled Gangliosides of Hippocampus in Offspring.

Radiolabeled gangliosides were extracted from the hippocampus of control (C) and ethanol (E) pups at 10, 17 and 24 days of age. A mixture of radiolabeled gangliosides from a control (C) and ethanol (E) rat of the same age was spotted on a HPTLC plate and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by exposing the plate to I<sub>2</sub> vapor in a glass tank. Individual gangliosides were scraped from the plate and solubilized in water and 10 ml of Aquasol. Radioactivity was determined by means of a liquid scintillation counter. Approximately 40,000 [<sup>3</sup>H] dpm (control) and 18,000 [<sup>14</sup>C] dpm (ethanol) were recovered from each lane. Results are expressed as the percentage of total DPM of ganglioside sialic acid DPM.

Each value represents the mean  $\pm$  the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

(--) indicates that these gangliosides were not detected.

TABLE A-X

## Effects of Maternal Ethanol Consumption

on % Distribution of Sialic Acid among Individual Radiolabeled Gangliosides of Hippocampus in Offspring

Ganglioside	10 Days		17 Days		24 Days	
	Control	Ethanol	Control	Ethanol	Control	Ethanol
GQ1b	12.5±1.7	12.2±1.8	10.4±1.3	10.3±1.0	4.2±0.5	4.9±0.6
GT1b	25.1±4.2	25.3±2.4	31.5±2.0	34.3±3.4	33.5±4.3	38.0±4.2
GT1a	6.7±2.6	3.8±1.6*	1.6±0.7	1.6±0.6		
GD1b	11.6±0.3	9.1±0.7*	15.9±1.6	15.8±0.6	14.0±2.3	13.7±2.9
GD2	---	---	---	---	---	---
GD1a	28.8±2.0	33.4±2.0*	26.7±1.2	28.9±2.4	30.1±1.7	27.5±1.8
GD3	15.3±2.2	16.2±1.8	13.6±1.2	9.8±1.6*	5.7±0.6	4.5±0.3*
GM1					12.6±1.4	11.5±0.4
GM3	---	---	---	---	---	---
GM4	---	---	---	---	---	---

Each value represents the mean ± the standard deviation of six animals.

A \* indicates that values from control and ethanol pups from the same groups are different at  $p < 0.05$ .

TABLE A-XI : Effects of Maternal Ethanol Consumption on The % distribution of Sialic Acid among Individual Radiolabeled Gangliosides of Corpus Striata in Offspring.

Radiolabeled gangliosides were extracted from the corpus striata of control (C) and ethanol (E) pups at 10, 17 and 24 days of age. A mixture of radiolabeled gangliosides from a control (C) and ethanol (E) rat of the same age was spotted on a HPTLC plate and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by exposing the plate to  $I_2$  vapor in a glass tank. Individual gangliosides were scraped from the plate and solubilized in water and 10 ml of Aquasol. Radioactivity was determined by means of a liquid scintillation counter. Approximately 20,000 [ $^3H$ ] dpm (control) and 4,000 [ $^{14}C$ ] dpm (ethanol) were recovered from each lane. Results are expressed as the percentage of total DPM of ganglioside sialic acid DPM.

Each value represents the mean  $\pm$  the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

(--) indicates that these gangliosides were not detected.

TABLE A-XI

## Effects of Maternal Ethanol Consumption

on % Distribution of Sialic Acid among Individual Radiolabeled Gangliosides of Corpus Striata in Offspring

Ganglioside	10 Days		17 Days		24 Days	
	Control	Ethanol	Control	Ethanol	Control	Ethanol
GQ1b	5.6±1.1	5.1±1.8	3.3±0.9	4.0±0.8	5.0±0.7	6.6±0.6*
GT1b	21.5±2.2	25.9±1.4*	18.9±1.4	23.5±0.9*	28.9±1.5	33.1±5.6
GT1a	3.1±0.3	2.0±0.5*				
GD1b	13.9±3.0	12.7±2.8	15.5±1.1	14.7±2.1	11.7±0.7	11.9±1.1
GD2	---	---	---	---	---	---
GD1a	35.7±2.7	34.8±3.1	42.3±2.5	40.6±1.1	37.2±3.1	35.4±2.4
GD3	5.8±0.9	5.6±0.9	6.3±0.8	4.2±1.0*	3.5±0.7	1.9±0.8*
GM1	14.6±2.2	13.8±2.1	13.8±2.3	13.1±1.4	13.7±2.5	11.2±2.1
GM3	---	---	---	---	---	---
GM4	---	---	---	---	---	---

Each value represents the mean ± the standard deviation of six animals.

A \* indicates that values from control and ethanol pups from the same groups are different at  $p < 0.05$ .

TABLE A-XII : Effects of Maternal Ethanol Consumption on The % distribution of Sialic Acid among Individual Radiolabeled Gangliosides of Hypothalamus in Offspring.

Radiolabeled gangliosides were extracted from the hypothalamus of control (C) and ethanol (E) pups at 10, 17 and 24 days of age. A mixture of radiolabeled gangliosides from a control (C) and ethanol (E) rat of the same age was spotted on a HPTLC plate and the gangliosides were separated in Methyl Acetate/Propanol/Chloroform/Methanol/0.25% aqueous KCl (25:20:20:20:17 v/v) solvent system. Authentic ganglioside standards were run on the same plate. Gangliosides were visualized by exposing the plate to I<sub>2</sub> vapor in a glass tank. Individual gangliosides were scraped from the plate and solubilized in water and 10 ml of Aquasol. Radioactivity was determined by means of a liquid scintillation counter. Approximately 10,000 [<sup>3</sup>H] dpm (control) and 2,500 [<sup>14</sup>C] dpm (ethanol) were recovered from each lane. Results are expressed as the percentage of total DPM of ganglioside sialic acid DPM.

Each value represents the mean ± the standard deviation of six animals.

A \* indicates that the values from control (C) and ethanol (E) animals from the same group are statistically significant using the Student's 't' test.

(--) indicates that these gangliosides were not detected.

TABLE A-XII

## Effects of Maternal Ethanol Consumption

on % Distribution of Sialic Acid among Individual Radiolabeled Gangliosides of Hypothalamus in Offspring

Ganglioside	10 Days		17 Days		24 Days	
	Control	Ethanol	Control	Ethanol	Control	Ethanol
GQ1b	13.0±2.1	17.1±4.0	17.7±1.1	16.7±1.8	11.6±0.3	12.0±0.1
GT1b	22.4±0.9	20.2±0.4*	27.4±0.6	31.7±1.7*	37.1±1.1	38.1±0.7
GT1a	---	---	---	---	4.6±0.8	4.5±0.7
GD1b	12.3±0.9	9.4±2.8	17.1±0.5	15.0±0.5*	11.3±0.5	11.4±0.5
GD2	---	---	---	---	---	---
GD1a	44.3±2.5	44.6±3.5	26.7±0.7	28.8±2.6	20.8±1.1	22.8±2.1
GD3					5.3±0.7	3.9±0.1*
GM1	8.1±1.1	8.7±2.8	11.3±1.0	7.8±1.4*	9.3±0.5	7.3±1.3*
GM3	---	---	---	---	---	---
GM4	---	---	---	---	---	---

Each value represents the mean ± the standard deviation of six animals.

A \* indicates that values from control and ethanol pups from the same groups are different at  $p < 0.05$ .



TABLE A-XIII  
Effects of Maternal Ethanol Consumption  
on Sialic Acid Concentration of Brain Regions in Offspring

Brain Region	<u>mg sialic acid per g of tissue</u>					
	10 Days		17 Days		24 Days	
	Control	Ethanol	Control	Ethanol	Control	Ethanol
Cerebellum	0.47±0.07	0.47±0.03	0.55±0.13	0.57±0.03	0.85±0.15	0.81±0.11
Cerebral Cortex	0.47±0.5	0.51±0.06	0.52±0.06	0.55±0.1	0.49±0.17	0.52±0.18
Brain Stem	1.00±0.11	0.70±0.19	0.75±0.19	0.83±0.13	0.81±0.19	0.86±0.15
Hypothalamus	0.77±0.1	0.72±0.1	1.00±0.26	1.18±0.71	1.60±0.5	1.60±0.5
Hippocampus	0.87±0.2	0.75±0.02	0.91±0.37	0.81±0.35	1.99±0.83	1.44±0.15
Corpus Striatum	0.79±0.08	0.71±0.19	1.00±0.18	0.89±0.16	0.86±0.26	0.92±0.44

Each value represents the mean ± the standard deviation of six animals.

## APPROVAL SHEET

The dissertation submitted by Antonio Blavatsky Caesar Noronha has been approved by the following committee:

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

November 22, 1982  
Date

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